

AD _____

GRANT NUMBER DAMD17-94-J-4138

TITLE: Role of Integrin Related Tyrosine Kinases in Growth Control of Normal and Tumorigenic Human Mammary Epithelium

PRINCIPAL INVESTIGATOR: Doctor George Plopper
Vitro Quaranta, M.D.

CONTRACTING ORGANIZATION: The Scripps Research Institute
La Jolla, California 92037

REPORT DATE: November 1997

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

19980416 143

DTIC QUALITY INSPECTED 4

REPORT DOCUMENTATION PAGE

*Form Approved
OMB No. 0704-0188*

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY <i>(Leave blank)</i>			2. REPORT DATE November 1997		3. REPORT TYPE AND DATES COVERED Final (1 Jul 94 - 30 Nov 97)		
4. TITLE AND SUBTITLE Role of Integrin Related Tyrosine Kinases in Growth Control of Normal and Tumorigenic Human Mammary Epithelium			5. FUNDING NUMBERS DAMD17-94-J-4138				
6. AUTHOR(S) Doctor George Plopper Vitro Quaranta, M.D.							
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The Scripps Research Institute La Jolla, California 92037				8. PERFORMING ORGANIZATION REPORT NUMBER			
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Commander U.S. Army Medical Research and Materiel Command Fort Detrick, Frederick, Maryland 21702-5012				10. SPONSORING/MONITORING AGENCY REPORT NUMBER			
11. SUPPLEMENTARY NOTES							
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited				12b. DISTRIBUTION CODE			
13. ABSTRACT <i>(Maximum 200)</i> We examined the role of the extracellular matrix protein laminin-5 (Ln-5) in static adhesion and migration of normal (HUMEC), immortalized (MCF-10A) and malignant breast epithelial cells that exhibit different degrees of metastatic potential (MDA-MB-435>MDA-MB-231>MCF-7). HUMEC, MCF-10A, and MCF-7 cells adhered to purified Ln-5 through the $\alpha 3\beta 1$ integrin receptor in rapid adhesion assays. While HUMEC and MCF-10A cells remained statically adherent, MCF-7 cells migrated constitutively on Ln-5 in <i>in vitro</i> assays. MDA-MB-231 and MDA-MB-435 cells bound and migrated on Ln-5 through a $\beta 1$ integrin receptor that is refractory to antibodies that block the function of $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, and αV integrin subunits. Direct stimulation of the $\alpha 3\beta 1$ integrin receptor with the $\beta 1$ stimulating antibody TS2/16 is sufficient to induce migration of MCF-10A cells on Ln-5, and this migration is blocked by inhibitors of G proteins (pertussis toxin), adenylate cyclase (SQ22536), and protein kinase A (H-89), suggesting that these signalling proteins may form an integrin-associated signalling pathway in MCF-10A cells. These inhibitors also block constitutive migration of the three malignant breast cell lines, suggesting that this pathway may play a role in the spread of malignant cells in breast cancer.							
14. SUBJECT TERMS Breast Cancer Integrins, laminins, cell migration, G proteins, cell signalling				15. NUMBER OF PAGES 51			
				16. PRICE CODE			
17. SECURITY CLASSIFICATION OF REPORT Unclassified		18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified		19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified		20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

Where copyrighted material is quoted, permission has been obtained to use such material.

Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

EP Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

PL In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.


PI / Signature 7/1/97
Date

Table of Contents

Front Cover	1
SF298 Report Documentation Page	2
Foreword	3
Table of Contents	4
Introduction	5
Body	8
Conclusions	18
References	22
Appendix (Tables, Figure Legends)	31
Publications/bibliography	36

Introduction.

Breast cancer progresses in two general stages. It begins as a carcinoma of clonal, hyperproliferating cells that may remain confined to breast ducts for several years as a benign, primary tumor (1). Benign tumors are rarely lethal; removal of these tumors by surgical and/or radiological methods is often sufficient to cure the disease if it is caught at this stage. The second stage, malignancy, is significantly more dangerous and is difficult to treat successfully. Preventing progression to the malignant stage is thus important in controlling the morbidity of breast cancer.

Malignancy begins when breast tumor cells acquire the ability to penetrate the surrounding basement membrane layer of extracellular matrix (ECM) proteins and migrate into nearby blood and lymphatic vessels (2), where they disseminate and continue proliferating to form secondary tumors (metastases). Acquisition of an aggressively migratory phenotype is a defining characteristic of malignant cancer cells (3).

The cellular machinery controlling migration is only partly understood. Cytoplasmic projections (pseudopodia) extend out from the body of a migrating cell in the direction of migration, forming a "leading edge" behind which the remainder of the cell follows (4). Pseudopodia are enriched in proteins thought to control the direction and rate of cell migration (5). Chief among these proteins are the integrin receptors. Integrins are heterodimeric, cell surface receptors that bind to the ECM proteins through which cells must pass as they migrate. Currently, there are 16 known α subunits and 8 β subunits that combine to form over 20 different receptors (6). Integrins also form clusters (focal adhesions) that control cell shape by connecting the inner surface of the plasma membrane with the actin cytoskeleton. Integrin-mediated cell migration is thus viewed as a coordinated series of adhesion/de-adhesion events at the cell surface, accompanied by changes in cell shape as the cell crawls through the ECM (4).

The ability to migrate is shared by virtually all cells. What distinguishes normal from malignant cells is that normal cells possess the ability to selectively migrate at defined times in response to defined stimuli. It is assumed that the mechanisms controlling normal cell migration fail in malignant cells, such that these cells move regardless of external stimuli (7). Reports of alterations in the expression of integrins and their ECM ligands in breast cancer cells abound (1,2,8-29), suggesting that these proteins may play an important regulatory function in malignant cell migration. How this regulation may be manifested at the functional level is unknown.

Recent evidence suggests that members of the laminin family of ECM proteins, as well as their integrin receptors, may play an especially important role in controlling breast cell function. Both laminin-1(30) and laminin-5 (Fig. 1) are abundantly expressed in breast basement membrane, and have been shown to play a major role in controlling growth, migration, and differentiation of breast epithelial cells. For example, laminin-5 supports branching morphogenesis and stable, static cell adhesion and formation of hemidesmosomes in breast cells via the $\alpha 6\beta 4$ integrin (31), yet is also expressed in regions of metastatic tumors where malignant cells are invading surrounding tissues (32). How breast cells switch from stationary to migratory phenotypes on laminin-5 is unknown.

Integrins are signalling receptors (6). At present, at least five different classes of signalling proteins have been associated with integrin receptors. These include protein tyrosine kinases, serine/threonine kinases, lipid kinases, ion channels, and members of the rho family of small molecular weight GTP binding proteins (33). How these signalling molecules combine to form signalling pathways is only recently being revealed. At present, very little is known about the signals generated by laminin-5 binding integrins or how these integrins control cellular behavior. However, changes in integrin-associated signalling proteins are observed in malignant cells. For example, many oncogenes are known to encode signalling proteins active in integrin-associated pathways, and increased expression of these and other integrin-associated signalling proteins has been found in malignant breast tumors (34-36). Overexpression and/or constitutive activation of these molecules leads to an increase in migration *in vitro* and increased metastatic capability in animal models (37-42).

Despite these enticing leads, no migration-specific signalling events have been positively associated with malignant breast cells. One signalling pathway that has been shown to affect migration of other cell types is that mediated by the second messenger molecule cyclic adenosine monophosphate (cAMP). cAMP is produced from ATP by the enzyme adenylate cyclase, which in turn is controlled by several classes of stimulatory and inhibitory heterotrimeric G proteins and their associated receptors (43). The activity of some of these G proteins can be modified by bacterial toxins that act as ADP ribosylation factors. For example, members of the G_s class of G proteins stimulate adenylate cyclase activity and are themselves susceptible to activation by cholera toxin, while members of the G_i class inhibit adenylate cyclase and are in turn inhibited by pertussis toxin. The net effect of both toxins is to ultimately induce an increase in intracellular cAMP to superphysiological levels, which ultimately inhibits downstream targets such as cAMP-dependent protein kinase (protein kinase A) (44). Despite their apparently redundant effects, cholera toxin and pertussis toxin inhibit distinct intracellular signalling pathways and are routinely employed to distinguish G protein linked signalling pathways upstream of cAMP production. Migration of many cell types, including lymphocytes, is inhibitable by cholera and pertussis toxins (45,46). To date, no evidence has been presented linking cholera toxin- and pertussis toxin-sensitive signalling pathways to integrin signalling.

In this project we examined the adhesion and migration on laminin-5 of normal (HUMEC) and spontaneously immortalized (MCF-10A) breast epithelial cells, and breast epithelial cells that exhibit different degrees of malignancy (MDA-MB-435>MDA-MB-231>MCF-7). We found that normal breast epithelial cells from primary cultures adhered to laminin-5 via the integrin $\alpha 3\beta 1$, but remained statically adherent and displayed no motility in two *in vitro* motility assays. A non-tumorigenic cell line, MCF-10A, also displayed $\alpha 3\beta 1$ -dependent static adhesive behavior and no migration on laminin-5. In contrast, MCF-7 cancer cells used $\alpha 3\beta 1$ integrin to adhere to laminin-5, but were constitutively migratory. The more malignant lines MDA-MB-231 and MDA-MB-435 also migrated vigorously on laminin-5, but used a distinct, unidentified $\beta 1$ integrin. In an effort to understand the mechanisms by which migration is controlled in normal and malignant breast cells, we have focused on identifying signalling pathways that control migration in these cells. Direct stimulation of the $\alpha 3\beta 1$ integrin receptor with the $\beta 1$ stimulating antibody TS2/16 is sufficient to induce

migration of MCF-10A cells on laminin-5. TS2/16-stimulated migration on laminin-5 is blocked by inhibitors of G proteins (pertussis toxin), adenylate cyclase (SQ22536), and protein kinase A (H-89), suggesting that these signalling proteins may form an integrin-associated signalling pathway in MCF-10A cells. These inhibitors also block constitutive migration of the three malignant breast cell lines on laminin-5, suggesting that this pathway may play a role in the spread of malignant cells in breast cancer.

Materials and Methods.

Cells. HUMECs (ninth passage) were purchased from Clonetics (San Diego, CA), maintained in Mammary Epithelial Growth Medium (Clonetics) a serum-free defined medium, and used by passage 12. Cells were passaged using the Clonetics Reagent Pack as indicated by the manufacturer. MCF-10A cells were maintained and passaged in DFCI medium according to Band and Sager (47). MCF-7, MDA-MB-231, and MDA-MB-435 cells were maintained in RPMI supplemented with 10% fetal calf serum (Gemini, Irvine CA) and 2 mM glutamine/penicillin G (100 units/ml)/Streptomycin sulfate (100 µg/ml) (BioWhittaker, Walkersville MD), and routinely passaged using trypsin/EDTA (Biowhittaker). Rat 804G cells were passaged and maintained under conditions identical to those for MCF-7 cells except that DMEM was substituted for RPMI medium. All cells were maintained at 37°C in a humidified incubator containing 10% CO₂. 804G cell conditioned medium was collected after 3 days of culturing and was clarified by centrifugation at 1500 x g.

Reagents. Mouse laminin-1 was purchased from Collaborative Biomedical Products (Bedford, MA). Mouse monoclonal antibodies against integrin α3 (clone P1B5) and β1 (Clone P4C10) were purchased from Gibco (Gaithersburg, MD). Mouse monoclonal antibody clone P5D2 against β1 integrin was purchased from Chemicon (Temecula, CA). Mouse monoclonal antibody TS2/16 (in ascites form) was generously provided by Dr. Martin Hemler (Dana Farber Cancer Institute, Boston, MA). Anti-laminin-5 monoclonal antibody TR1 was produced in this laboratory (48). Both TS2/16 and TR1 were purified with a protein G affinity chromatography kit (Pierce). Anti-mouse Fc monoclonal antibody, adrenalin, bombesin, lysophosphatidic acid, 8-bromo-cAMP, dibutyryl cAMP, forskolin, and cholera toxin were purchased from Sigma. H-89 and calphostin C were purchased from Calbiochem. SQ22536 was purchased from Biomol (Plymouth Meeting, PA). Genistein was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Pertussis toxin was purchased from List Biological Laboratories, Inc. (Campbell, CA). Rat monoclonal antibody GoH3 against the α6 integrin was purchased from Immunotech (Westbrook ME). Function-blocking, mouse monoclonal antibodies against integrin α2 (clone P1E6), α3 (clone P1B5), α5 (clone P1D6) and β1 (Clone P4C10) were purchased from Gibco (Gaithersburg, MD). Mouse monoclonal antibody J143 against α3 integrin (49) was kindly provided by Dr. Yves Fradet (Memorial Sloan-Kettering Cancer Center, New York NY). Function-blocking, mouse monoclonal antibodies against integrin α1 (clone 5E8D9) and α4 (clone A4-PUJ1) were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Rat monoclonal antibody 69-6-5 against integrin αV was generated in this laboratory (50). Mouse antibodies MOPC 31c and monoclonal anti-vinculin as well as goat anti-mouse and anti-rat secondary antibodies coupled to fluorescein isothiocyanate were purchased from Sigma (St. Louis, MO). Mouse monoclonal antibodies FM3 and CM6 against laminin-5 were isolated and purified from ascites fluid as described elsewhere (48).

Adhesion assays. Untreated 96 cell plates (Sarstedt, Newton, NC) were coated for at least two hours at room temperature with rat or mouse laminin-1 (Gibco), human laminin-2 (placental laminin, Gibco), anti-laminin-5 monoclonal antibodies (all at 20 µg/ml) or with human fibronectin or human vitronectin (both at 40 µg/ml, Gibco). All proteins were diluted in 100 mM carbonate buffer, pH 9.3. Plates were then washed

twice with phosphate buffered saline (PBS) containing 0.2% Tween 20 (PBST) and blocked overnight at 4°C with blotto (5% nonfat dried milk in PBST). Following two washes with PBST, wells containing anti-laminin-5 antibodies were incubated for 1 hour at room temperature with 804G cell conditioned medium, thereby allowing for "capture" of soluble laminin-5, then washed twice with PBST. For CM6 antibody blocking experiments, wells were incubated with indicated concentrations of blocking antibodies diluted in blotto. As controls, wells were blocked with blotto alone, irrelevant antibody MOPC 31c, or anti-laminin-5 monoclonal antibody FM3. Wells were washed twice with PBST.

In some experiments, cells were allowed to attach to 804G cell matrix or wells coated with 804G cell conditioned medium. To prepare 804G cell matrix, cells were grown to confluence in Sarstedt 96 well plates, the culture medium was removed and the cells were washed in sterile PBS. The cells were removed according to the method of Gospodarowicz (51) by incubating them 2 x 5 minutes in 20 mM sterile NH₄OH. The wells were extensively washed with PBS and distilled water and blocked with blotto as described above. Additional wells were coated for 2 hours with 804G cell conditioned medium, then washed and blocked as described above.

Cells were collected by brief trypsinization, blocked with either serum-containing medium or Trypsin Inhibitor solution (Clonetics), washed twice with DMEM/1% bovine serum albumin, then plated (1.2×10^5 / well) in DMEM/1% bovine serum albumin/25 mM HEPES, pH 7.2. For anti-integrin antibody blocking experiments, cells were incubated at room temperature with blocking antibodies for 30 minutes before addition to plates; blocking antibodies were present during plating. Plates were kept at 37°C in a humidified incubator containing 10% CO₂ for 30 minutes. To remove unbound cells, wells were then filled with PBS and the plates were inverted in a tank of PBS and allowed to gently shake for 15 minutes. Excess PBS was absorbed from the wells by inverting plates on paper towels. Bound cells were fixed in 3% paraformaldehyde/PBS, then stained with 0.5% crystal violet in 20% methanol/80% H₂O. Wells were washed with water to remove excess dye, then cells were solubilized in 1% SDS and the amount of dye was quantitated using a Molecular Devices plate reader set to absorb at 595 nm.

Transwell haptotactic migration assays. Transwell filters (8.0 µm pore size, Costar, Cambridge MA) were coated for 4 hours with identical concentrations of extracellular matrix proteins used in adhesion assays, diluted in 100 mM carbonate buffer, pH 9.3. Separate filters were coated for 1 hour with mouse monoclonal antibody TR1, diluted in 100 mM carbonate buffer. Antibody-coated filters were blocked for 2 hours with blotto, then incubated for 1 hour with 804G cell conditioned medium, thereby allowing for "capture" of laminin-5 on the filter. For CM6 antibody blocking experiments, filters were incubated with indicated concentrations of blocking antibodies diluted in blotto. As a control, filters were blocked with blotto alone. Following two washes with PBST, filters were inverted and cells (6×10^4 cells/filter) were plated on the uncoated side in migration medium (DMEM/2 mM glutamine/1 mM sodium pyruvate). For anti-integrin antibody blocking experiments, cells were incubated with blocking antibodies in migration medium for 30 minutes before plating on filters. Antibodies were also present in migration medium throughout the migration assay. Cells were maintained at 37°C in a humidified incubator containing 10% CO₂ for 18 hours, then filters were

fixed and cells stained using the Diff-Quik stain kit (Baxter, McGaw Park, IL). The uncoated side of each filter was wiped with a cotton-tipped applicator to remove cells that had not migrated through the filter. Filters were then cut from their supports, mounted on slides and viewed under bright field optics. To quantitate migration, stained cells were counted in four fields (under 300 x magnification) from each of two filters for each condition. Results were expressed as the mean number of cells counted in each field ± the standard deviation.

Colloidal gold uptake motility assays. Colloidal gold motility assays were performed exactly as described by Albrecht-Buhler (52). Colloidal gold particles coated on glass coverslips were coated with laminin-5 affinity captured by TR1 as described for adhesion assays. As controls, coverslips coated with gold particles were blocked with blotto and incubated with 804G cell conditioned medium. Cells were collected as for cell adhesion assays and plated (5,000/well) in 6 well plates containing coated coverslips. After 18 hours, cells were fixed in 10% formalin/PBS, viewed under dark field microscopy and photographed using Kodak Gold 200 color print film. Migration was quantitated by digitally scanning prints of photographic images (Scanjet IIcx; Hewlett Packard, Palo Alto CA, USA), and computing the black area (displaced gold) in scanned images using Adobe Photoshop 3.0 (Adobe Systems, Inc., Mountain View, CA, USA) running on a Quadra 950 computer (Apple Computer, Inc., Cupertino, CA, USA.). The results were expressed as the ratio of displaced gold area to the total area of each image ± standard deviation (n=3).

Immunofluorescence Microscopy. Cells were plated for 2 hours in migration medium on glass coverslips coated with laminin-5 matrix (53), then washed briefly with PBS, fixed for 10 minutes in 3% paraformaldehyde/PBS, extracted with 0.01% Triton-X-100/PBS, rinsed twice with PBS, and blocked for 40 minutes with 10% goat serum/PBS. Primary antibodies were diluted in PBS and applied to coverslips for 1 hour. Following two washes with PBS, coverslips were incubated 1 hour with rabbit anti-mouse F(ab')2 secondary antibody coupled to fluorescein isothiocyanate (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), diluted 1:30 in PBS. Following two more washes with PBS, cells were incubated with a 1:10 dilution of rhodamine-coupled phalloidin (ICN Immunobiologicals, Costa Mesa, CA) for 40 minutes to stain filamentous actin. Stained coverslips were washed twice in PBS, mounted with Immunofluore mounting medium (ICN), viewed with a Axiophot I microscope equipped for epifluorescence (Zeiss, Germany), and photographed on Kodak T-max 400 film.

FACS analysis. Cells were typsinized, blocked, and washed as for adhesion assays, then washed twice with ice cold FACS buffer (Hanks Buffered Saline Solution containing 5% fetal calf serum and 0.02% NaN₃), with each wash followed by gentle centrifugation at 4°C (500x g). All subsequent steps were performed at 4°C. Cells were then incubated for 1 hour with anti-integrin antibodies diluted at the same concentrations used for adhesion and migration assays in FACS buffer, then washed twice with secondary antibody buffer (Hanks Buffered Saline Solution containing 5% goat serum and 0.02% NaN₃). Cells were incubated for one hour with goat anti-mouse or goat anti-rat secondary antibodies coupled to fluorescein isothiocyanate (diluted 1:128 or 1:200, respectively, in secondary antibody buffer), washed twice with FACS buffer, then analyzed on a Beckton-Dickinson FACScan flow cytometer. As a

control, cells were incubated with anti-mouse or anti-rat secondary antibodies only.

Polymerase chain reaction. Reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out as described previously.(54). Briefly, poly(A)+ RNA from HUMEC and MCF-7 cells was isolated using the Micro-FastTrack mRNA isolation kit (Invitrogen, San Diego CA). First-strand cDNA was prepared by using oligo(dT) primers and the RT-PCR kit (Stratagene, San Diego). Oligonucleotides were synthesized with a Cyclone Plus DNA synthesizer (Millipore, Bedford MA). Primers for $\alpha 3$ were: 3484, 5'-AAGCCAAGTCTGAGACTGTG-3'; and 3485, 5'-GTAGTATTGGTCCCGAGTCT-3', corresponding to nucleotides 2757-2776 and 3393-3413, respectively, of the human $\alpha 3$ sequence (55).

Immunoprecipitation. HUMEC and MCF-7 cells were surface labeled with biotin using Sulfo-NHS-LC-Biotin (Pierce, Rockford IL) as directed by the manufacturer. Surface labeled cells were solubilized on ice in PBS/2% Renex30 (Accurate Chemicals, Westbury NY)/1 mM PMSF/1 μ g/ml aprotinin/1 μ M leupeptin/1 μ M pepstatin. Lysates were precleared by adding sepharose beads coupled to goat anti-mouse IgG antibodies (Sigma) for 30 minutes at 4°C, and clarified by centrifugation at 4°C. $\alpha 3$ integrin subunits were immunoprecipitated from lysates by adding 10 μ l cell lysate, 5 μ l P1B5 antibodies and 10 μ l antibody-coupled agarose per sample to 200 ml immunoprecipitation buffer (50 mM Tris-HCl/0.5 M NaCl/1 mM CaCl₂/ 1 mM MgCl₂/0.1% Tween 20) and rocking overnight at 4°C. Agarose beads were pelleted by centrifugation and washed 5 times with immunoprecipitation buffer, then boiled in reducing sample buffer. The supernatants were separated by SDS-PAGE, transferred to PVDF membranes, blocked overnight with 0.1% Tween 20/0.2% gelatin/PBS at 4°C, then incubated with streptavidin conjugated to horseradish peroxidase (Boehringer Mannheim, Indianapolis, IN) diluted 1:4000 in blocking buffer for 1 hour. Membranes were washed 3x with PBS/0.2% Tween 20 and developed using the ECL chemiluminescence kit according to the manufacturer (Amersham, Arlington Heights, IL).

Confocal immunofluorescent detection of laminin-5 in rat mammary gland. Immunofluorescent labeling for laminin-5 was performed on 8 μ m thick cryostat sections prepared from snap frozen rat mammary gland. Sections were mounted on glass slides, dried at room temperature (RT) for 1 hour, fixed in freshly made 4% formaldehyde (from paraformaldehyde) for 20 minutes at 4°C, permeabilized in 0.1% saponin for 5 minutes at room temperature, and then incubated in 50 mM glycine in PBS to saturate reactive groups generated by formaldehyde fixation. Nonspecific binding was blocked by incubation of sections in PBS containing 2% donkey normal serum (DS) (Jackson Immunoresearch Lab. Inc., West Grove, PA, USA) and 1% BSA (fraction V; Sigma Immunochemicals, St. Louis, MO, USA) for 1 hour at room temperature. Following extensive washes in PBS (0.2% DS, 0.1% BSA), sections were incubated for 1 hour at room temperature with 20 μ g/ml of either CM6, or isotype matched mouse IgGs, used as control reference for specificity of CM6 antibody. After several washes in PBS (0.2% DS, 0.1% BSA), sections were challenged with an FITC-conjugated affinity-purified donkey anti-mouse secondary antibody (Jackson Immunoresearch Lab. Inc., West Grove, PA, USA) for 1 hour at room temperature. Following six washes of 5 minutes each with PBS (0.2% DS, 0.1% BSA), sections were mounted in slow fade medium and viewed on a Zeiss Axiovert 35M microscope.

(Carl Zeiss, Thornwood, NY, USA), using a 40X 1.3 NA objective lens, equipped with a laser scanning confocal attachment (MRC-1024, Bio-Rad Laboratories, Cambridge, MA, USA). Fluorescent images were collected by using an argon/krypton mixed gas laser. Composite images were generated using Adobe Photoshop, and printed on a Tektronix Phaser II-SDX (Tektronix, Inc., Beaverton, OR, USA).

cAMP determination. Cells were collected by brief trypsinization, blocked with trypsin inhibitor, washed in DMEM, and incubated at 37°C in migration medium containing 1 mM isobutylmethylxanthine (Sigma) to block phosphodiesterase activity. After 30 minutes, cells were collected by centrifugation, and 1×10^6 cells per sample were suspended in media containing the indicated agonists. As controls, cells were suspended in DMEM/1mM isobutylmethylxanthine alone or plated in 35 mm dishes affinity coated with laminin-5. After 30 minutes, suspended cells were collected by centrifugation, and the cell pellet solubilized in ice cold cAMP extraction solution (95% ethanol, 5% 0.1 N HCl). After one wash with phosphate buffered saline to remove nonadherent cells, cells plated on laminin-5 were scraped directly into ice cold cAMP extraction buffer. All samples were kept on ice in cAMP extraction buffer for 2 hours, then centrifuged to pellet precipitated protein. Pelleted protein was suspended in 0.1 N NaOH and protein amounts were determined with the BCA microassay (Pierce). Supernatants were evaporated and cAMP measured using a cAMP EIA kit (Perseptive Diagnostics, Inc., Cambridge MA) as directed by the manufacturer. cAMP amounts were normalized to total protein in each sample and expressed as fmol/ μ g protein.

Results.

Laminin 5 is expressed in breast.

To determine the distribution of laminin-5 in the mammary gland, we immunostained cryostat sections from snap-frozen mammary gland of a lactating rat with CM6, a rat-specific monoclonal antibody against the $\alpha 3$ chain of laminin-5 (48) (Fig. 1). Sections were observed at a BioRad MRC 1024 confocal system. We found a bright, though non-contiguous, fluorescent immunoreactivity near the basal (basolateral) pole of luminal cells forming the mammary ductal epithelium with antibody CM6 (Fig. 1, panels A, B). Higher power fields (Fig. 1, panel C) revealed that in some instances, laminin-5 staining completely surrounded basal (myoepithelial) epithelial cells. Control sections stained with isotype matched mouse IgGs were negative (Fig. 1, panel D). These observations demonstrate that laminin-5 is present in basement membranes of mammary epithelium, in direct contact with epithelial cells.

Normal, immortalized, and MCF-7 malignant human breast epithelial cells bind laminin-5 through the $\alpha 3\beta 1$ integrin.

To investigate a potential role for laminin-5 in the organization of breast epithelium, we tested primary human breast epithelial cells (HUMEC); a spontaneously immortalized, non-malignant breast epithelial cell line (MCF-10A) (56); and three malignant breast epithelial cell lines (MCF-7, MDA-MB-435, MDA-MB-231) (57-59) in rapid (30 minute) adhesion assays. HUMECs and MCF-10A cells adhered efficiently to wells coated with laminin-5 (Fig. 2). Rapid adhesion of HUMECs to all other ECM molecules tested was relatively poor (Fig. 2). The most active of these was laminin-2, which supported approximately 20% as much adhesion as laminin-5 (Fig. 2). As controls, plates coated with antibody, the blocking agent "blotto", or DMEM/10% FCS did not promote adhesion. In contrast to HUMECs, the MCF-10A, MCF-7, 435, and 231 cells adhered well to laminin-5, laminin-2 and fibronectin (Fig. 2).

To investigate the contribution of specific integrins to normal and malignant cell adhesion to laminin-5, we used blocking monoclonal antibodies that recognize integrin chains reported to be involved in binding to laminins: P1E6 against $\alpha 2$ (60), P1B5 against $\alpha 3$ (60), GoH3 against $\alpha 6$ (61), and P4C10 against $\beta 1$ integrins (62). In rapid adhesion assays, antibodies against $\alpha 3$ and $\beta 1$ integrin subunits reduced adhesion of HUMEC, MCF-10A, and MCF-7 cells by greater than 90% (Fig. 3). In contrast, antibodies against $\alpha 2$ and $\alpha 6$ had no inhibitory effect on the adhesion of either cell type to laminin-5.

We used RT-PCR analysis and immunoprecipitation to establish that HUMEC and MCF-7 cells express the A isoform of the $\alpha 3$ integrin receptor. Following RT-PCR we detected a 570 bp band corresponding to the $\alpha 3A$ variant in both HUMECs and MCF-7 cells (Fig. 4a). We detected no 426 bp band corresponding to the $\alpha 3B$ variant in either cell type. Immunoprecipitation of cell surface, biotin-labeled $\alpha 3$ integrin with the P1B5 antibody revealed that both cell types expressed $\alpha 3$ subunits of virtually identical size (Fig. 4b). FACS analyses (Table 1) revealed that both cell types expressed nearly identical amounts of $\alpha 3$ integrin subunits on their surface. We conclude that normal and relatively weakly malignant MCF-7 cells utilize the same integrin receptor, $\alpha 3A\beta 1$, to bind laminin-5.

Both MDA-MB-231 and MDA-MB-435 cell lines were inhibited from binding laminin-5

by antibodies against the $\beta 1$ integrin subunit, but antibodies against the $\alpha 2$, $\alpha 3$, and $\alpha 6$ integrin subunits failed to significantly interfere with adhesion in these cell types (Fig. 4). Consistent with earlier studies (19), in control experiments the same concentrations of GoH3 antibody were able to block MDA-MB-231 and MDA-MB-435 cell adhesion to laminin-1, demonstrating that the $\alpha 6\beta 1$ and/or $\alpha 6\beta 4$ integrin is functionally expressed on the surface of these cells (not shown). FACS analysis revealed that $\alpha 2$, $\alpha 3$, and $\alpha 6$ integrins were expressed on the surface of these cells (Table 1). Additional experiments using higher concentrations of anti- α subunit inhibitory antibodies (up to 50 μ g/ml) and inclusion of antibodies that block the function of $\alpha 1$, $\alpha 4$, $\alpha 5$, and αV integrins failed to inhibit adhesion of either cell type (not shown). We conclude that the MDA-MB-231 and MDA-MB-435 cell lines differ from HUMEC, MCF-10A, and MCF-7 cells in that they do not use the $\alpha 3\beta 1$ integrin to bind laminin-5; rather, they use a $\beta 1$ integrin that is resistant to $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, and αV function blocking antibodies.

This observation suggested that MDA-MB-231 and MDA-MB-435 cells might bind different portions of laminin-5 compared to normal or MCF-7 cells. We recently found that the anti-laminin-5 monoclonal antibody CM6 disrupts cell adhesion and hemidesmosome formation in keratinocyte cell lines plated on laminin-5 enriched extracellular matrix preparations (63). When tested in rapid adhesion assays, laminin-5 captured by CM6 antibody did not support adhesion of any of the five cell lines (Fig. 5). Identical results were obtained when CM6 was added to wells containing laminin-5 affinity captured by the antibody TR1 (not shown). CM6 is a slightly more efficient capturing agent than is TR1¹, suggesting that adhesion wells coated with CM6 contained at least as much laminin-5 as wells coated with TR1; yet, these wells supported no cell adhesion whatsoever (Fig. 5). Based on these results, we conclude that both normal and malignant cells bind the same region of laminin-5, regardless of the integrin receptor employed.

MCF-7, but not HUMEC or MCF-10A cells, use $\alpha 3\beta 1$ integrin to spontaneously migrate on laminin-5.

To measure random cell motility following adhesion to laminin-5, cells were plated on colloidal gold particles coated with antibody-captured laminin-5. As cells randomly migrated they displaced the gold particles leaving behind a dark trail easily viewed under dark field optics (52). MCF-7 cells were actively motile on laminin-5 coated gold particles. They formed rounded, multicellular aggregates, appeared to collect laminin-5-coated gold particles on their surfaces, and were loosely adherent to the glass. On control, BSA coated particles, they instead spread, migrated poorly, and did not aggregate (Fig. 6). MDA-MB-435, and MDA-MB-231 cells behaved similarly in this assay (not shown). In contrast, HUMECs migrated on laminin-5-coated gold at background levels, approximately equal to that on control, BSA coated gold particles (Fig. 6).

The differential behavior of non-migratory (MCF-10A) and migratory (MCF-7) cells plated on laminin-5 was reflected by the overall cell morphology and organization of the actin cytoskeleton in these two cell types. Within two hours after plating on

¹ M. Fitchmun et al., manuscript submitted.

laminin-5, MCF-10A cells adopted an overall cobblestone-like shape ringed by short filopodial extensions and formed an elaborate network of actin microfilaments including a circumferential microfilament bundle near the cell periphery (Fig 7, panel a). In contrast, MCF-7 cells adopted a more elongated shape and extended large cytoplasmic processes that contained considerably fewer actin bundles (Fig. 7, panel b), consistent with a significant reduction of actin bundle formation observed in malignant cells *in vivo* (2,64-67). In MCF-10A cells, the actin binding protein vinculin was localized by immunofluorescence microscopy to the entire cell periphery, principally in short filopodial extensions (Fig. 7, panel c), while in MCF-7 cells it concentrated only at the tips of filopodia (Fig. 7, panel d). Immunolocalization with the antibody J143 revealed that $\alpha 3$ integrin was concentrated in the filopodial extensions of MCF-10A cells (Fig. 7, panel e). In contrast, in MCF-7 cells $\alpha 3$ was concentrated in small foci randomly distributed throughout the cytoplasm (Fig. 7, panel f). These observations are consistent with the stationary and migratory phenotypes, respectively (68,69) that MCF-10A and MCF-7 adopt following adhesion to laminin-5.

To measure haptotactic migration on laminin-5, we plated normal and malignant cells on the upperside of Transwell filters (8 μm pore diameter) coated on the underside with laminin-5. Consistent with our colloidal gold migration assays, MCF-7, MDA-MB-435, and MDA-MB-231 cells migrated very efficiently through laminin-5 coated filters (Fig. 8). In contrast, HUMEC and MCF-10A cells did not: they were as migratory on laminin-5 coated filters as on control (BSA) coated filters (Fig. 8). Consistent with our cell adhesion data, migration of MCF-7 cells was inhibited by the anti-laminin-5 antibody CM6 and antibodies directed against $\alpha 3$ and $\beta 1$ integrins, while antibodies directed against $\alpha 2$ or $\alpha 6$ integrins had no effect (Fig. 8). Migration of MDA-MB-435, and MDA-MB-231 cells was inhibited only by CM6 and antibodies against $\beta 1$ integrin: none of the antibodies against α subunits blocked migration.

In summary, both normal and MCF-7 cells adhere efficiently to laminin-5 through the $\alpha 3\beta 1$ integrin receptor, while MDA-MB-435, and MDA-MB-231 cells use an unidentified, $\beta 1$ -containing receptor. However, HUMECs and MCF-10A cells were statically adherent, whereas MCF-7, MDA-MB-435, and MDA-MB-231 cells actively migrated on laminin-5.

Integrin activation with the antibody TS2/16 stimulates MCF-10A cell migration on laminin-5, but not on laminin-1.

In an effort to understand the mechanisms controlling migration of normal and malignant breast cells on lamainin-5, we next focused on defining factors that stimulated MCF-10A cell migration when added to the basal migration medium in a haptotaxis migration assay. When added to wells containing Transwell filters coated with laminin-5, the $\beta 1$ integrin-activating monoclonal antibody TS2/16 induced MCF-10A cell migration on laminin-5 in a dose-dependent manner (Fig. 9). The non-activating anti- $\beta 1$ antibody P5D2 did not induce migration. Addition of soluble mitogens (1% serum, bovine pituitary extract [40 $\mu\text{g}/\text{ml}$], recombinant human epidermal growth factor [12.5 nM], and insulin [1 $\mu\text{g}/\text{ml}$]) also stimulated MCF-10A cell migration, but this stimulation was not additive with TS2/16 treatment. (Fig. 9). Addition of the $\alpha 3$ integrin blocking antibody P1B5 completely inhibited TS2/16-stimulated migration.

Importantly, TS2/16 antibody activation did not stimulate MCF-10A cell migration on laminin-1 (Fig. 10), even though these cells did migrate when plated in complete growth medium containing serum and growth factors. This result suggests that induction of migration by direct integrin activation may be specific for laminin-5 binding integrins in this cell line.

TS2/16 stimulates an increase in intracellular cAMP via a pertussis toxin-sensitive signalling pathway.

To define the signalling mechanisms by which TS2/16 stimulated MCF-10A cell migration on laminin-5, we added inhibitors of known signalling molecules to antibody-stimulated cells in haptotaxis migration assays. We found that pertussis toxin, an inhibitor of the G_i class of heterotrimeric G proteins, blocked TS2/16-stimulated migration of MCF-10A cells, while cholera toxin, an activator of Gs G proteins, did not (Fig. 11). In control experiments, both toxins blocked serum-stimulated migration (Fig. 11). These results suggest that TS2/16 stimulates MCF-10A cell migration by activating a signalling pathway containing the pertussis toxin-sensitive (G_i) subclass of heterotrimeric G proteins.

Heterotrimeric G_i proteins regulate downstream signalling events by controlling the activity of adenylate cyclase, which produces cAMP. To determine the direct role of cAMP in MCF-10A cell migration on laminin-5, we increased intracellular cAMP levels with either non-hydrolyzable analogs of cAMP (8-bromo-cAMP, dibutyryl cAMP) or an inhibitor of phosphodiesterase (forskolin), which cleaves cAMP to form AMP. These agents stimulated MCF-10A cell migration on laminin-5 but were not additive with antibody stimulation in these cells, again suggesting that these two stimulants functioned in a common signalling pathway (Fig. 12). Inhibition of cAMP production with the adenylate cyclase inhibitor SQ22536 blocked migration of antibody-stimulated cells (Fig. 12) on laminin-5. Finally, an inhibitor of cAMP-dependent protein kinase (H-89) also blocked TS2/16 cell migration on laminin-5 (Fig. 12). Addition of cAMP analogs or forskolin failed to induce migration of these cells on laminin-1, again suggesting that this activation is specific to laminin-5-mediated migration.

These results suggested that TS2/16 signals through a pathway that uses intracellular cAMP as a second messenger. A 30 minute stimulation with TS2/16 induced a greater than two-fold increase in intracellular cAMP in non-adherent MCF-10A cells compared to untreated cells (Fig. 13). Addition of pertussis toxin also increased cAMP levels, although this increase was not additive with TS2/16 treatment (Fig. 13), suggesting that TS2/16 and pertussis toxin acted through a common signalling pathway.

cAMP is a common second messenger used in a variety of cellular signalling pathways. Not surprisingly, a rise in cAMP was not specific to TS2/16 stimulation in MCF-10A cells. Addition of control anti-b1 antibodies that block adhesion to laminin-5 (P4C10) or are non-functional (P5D2) also induced a rise in cAMP. This effect was exacerbated when these antibodies were clustered with a secondary, anti-mouse Fc antibody, which raised cAMP levels to that found in non-stimulated cells adherent to laminin-5 (Fig. 13).

Stimulation of MCF-10A cells with adrenalin, which activates cAMP production in other cell types, also induced significant increases in cAMP levels over the same time course (Fig. 13), although it did not stimulate MCF-10A cell migration on laminin-5 (not shown). Thus, cAMP is used as a second messenger in multiple signalling pathways in these cells, and while it appears to play a role in migration signalling, it is not used exclusively in this pathway.

Inhibition of the TS2/16 stimulated signalling pathway blocks constitutive migration of malignant breast cells on laminin-5.

To assess the relative importance of the TS2/16 stimulated signalling pathway in malignant transformation, we used MCF-7, MDA-MB-231 and MDA-MB-435 malignant cells, which constitutively migrate on laminin-5 in a minimal medium lacking soluble mitogens, serum, or integrin-activating antibodies. Migration of all three malignant cell lines on laminin-5 was blocked approximately 50% by pertussis toxin, while the adenylate cyclase inhibitor SQ22536, the PKA inhibitor H-89, and function blocking antibodies against β 1 integrin chain blocked migration completely (Fig. 14). Cholera toxin, genistein, and calphostin C also blocked migration in these cells 50-90%, suggesting that multiple signalling pathways mediating migration are activated in these cells.

Conclusions.

We examined the contribution of laminin-5 to adhesion and migration of normal and malignant breast epithelial cells in in vitro assays using integrin-specific inhibitory antibodies and purified laminin-5 under defined, serum-free conditions. One striking finding was that laminin-5 was more adhesive for normal breast epithelial cells than all other ECM molecules tested, including other ECM proteins present in breast basement membrane (30,70-72); this preferential adhesiveness was not true for the malignant cell lines. This is somewhat surprising because laminin-1 in particular has been reported to play a significant role in breast cell growth and differentiation in in vitro models (73). These findings, combined with the observation that laminin-5 supports branching morphogenesis of MCF-10A cells in vitro (31), our localization of laminin-5 to the basement membrane of normal rat breast gland (Fig. 1), and detection of laminin-5 in secreted matrix of HUMEC and MCF-10A cells², argue strongly that preferential adhesion to laminin-5, particularly through the $\alpha 3\beta 1$ integrin, plays a critical role in maintenance of normal breast epithelium.

Although the integrin receptor $\alpha 6\beta 4$ functions as a receptor for laminin-5 (74) and is expressed in breast epithelium (75), our data suggest that it does not play a role in rapid (30 minute) adhesion to laminin-5 or migration on laminin-5 in these cells. However, these data do not discount the possibility that $\alpha 6\beta 4$ may contribute to formation of long-term, stable adhesion complexes such as hemidesmosomes in these cells (76).

Integrin-mediated adhesion to laminin-5 appears to play a significant role in tumorigenesis. Laminin-5 has been localized to invading cancer cells in breast and other organs (32,77), and malignant MCF-7 cells secrete laminin-5 and incorporate it into their basement membrane in culture (G.E. Plopper, unpublished). Our observation that laminin-5 stimulates both random motility and haptotactic migration through the $\alpha 3\beta 1$ integrin in MCF-7 cells and through a different, unidentified integrin(s) in MDA-MB-435 and MDA-MB-231 cells suggests that laminin-5 may also play a role in invasion and metastasis.

Interestingly, all five cell lines failed to attach to laminin-5 when plated in the presence of the anti-laminin-5 monoclonal antibody CM6. This was true whether CM6 was used as a blocking agent on a laminin-5 enriched ECM matrix (63), affinity captured laminin-5 (Fig. 5) or as a capturing agent itself. CM6 recognizes an epitope on the globular tail portion of the $\alpha 3$ chain of laminin-5 (48,63). The results obtained from our adhesion assays suggest that at least two distinct integrin receptors adhere to laminin-5, via a common epitope on the $\alpha 3$ chain of laminin-5 defined by CM6. This is consistent with the observation that a monoclonal antibody, BM165, directed against the $\alpha 3$ chain of human laminin-5, also blocks cell adhesion (78).

How can such divergent cellular phenotypes as static adhesion in normal and immortalized cells and spontaneous migration in malignant cells result after binding laminin-5 through the same receptor, the integrin $\alpha 3\beta 1$? Although previous studies have identified changes in expression of integrin subunits in malignant tumors, no

²G.E. Plopper, unpublished observations.

conclusive marker for malignant transformation in breast has been identified. Inherent in many of these studies is the assumption that these integrins are fully and/or equally functional when expressed on the cell surface. As a result, integrins that are expressed at approximately equal levels in normal and malignant cells may be overlooked as potential participants in the mechanism of malignant transformation. Instead, we hypothesize that the $\alpha 3\beta 1$ integrin may function differently in normal and malignant cells, independent of surface expression level, and that this functional difference may be revealed at the level of integrin and actin cytoskeletal organization. Consistent with their behavior in migration assays, we found that MCF-10A and MCF-7 cells adopted significantly different morphologies following adhesion to laminin-5, coincident with a marked difference in $\alpha 3$ integrin, vinculin, and actin microfilament distribution in these cells. In particular, we saw significant overlap in the distribution of these proteins in MCF-10A cells, especially in structures resembling classical focal adhesion complexes at the cell periphery, but we saw little overlap in MCF-7 cells. That $\alpha 3$ integrin supports adhesion and migration of MCF-7 cells on laminin-5 but is not localized to focal adhesion complexes suggests that it may instead be clustered in so-called "point contacts" or "dot contacts" found in migratory astrocytes (79). As these contacts typically do not contain vinculin or actin (79), this may explain the disparate localization of these proteins in MCF-7 cells.

We therefore propose that $\alpha 3\beta 1$ integrin-mediated adhesion to laminin-5, with resulting formation of stress fibers and discrete focal adhesion complexes, no haptotactic migration, and no random motility, may be defining characteristics of normal breast epithelium. How this organization is disrupted during malignant progression is unclear. One possibility is that cytoskeletal changes result from corresponding changes in the activation states of the receptor, either directly via alterations in association with cytoskeletal elements in focal adhesion complexes, or indirectly through alterations in downstream chemical signalling pathways.

When the five cell lines used in this study are ranked according to growth characteristics in vitro and previously reported metastatic potential, and scored for behaviors on laminin-5 such as rapid adhesion, static adhesion, integrin receptor, random motility, and haptotactic migration, a striking pattern emerges (Table 2): While all cells share the ability to bind laminin-5, the preference of laminin-5 as an adhesive substrate decreases as cells become more malignant. Concomitant with this rise in indiscriminate adhesion is the appearance of constitutive migration and a conversion from the $\alpha 3\beta 1$ integrin to a different $\beta 1$ integrin as the laminin-5 receptor. Interestingly, these changes do not occur simultaneously: MCF-7 cells use $\alpha 3\beta 1$ as a laminin-5 receptor but they are constitutively migratory on laminin-5. Also, this conversion to a new integrin receptor occurs in the most malignant cell lines despite the fact that they express $\alpha 3$ integrin on their surface.

Based on these observations, we propose a simplified model for malignant progression in breast: Normal, non-malignant cells are statically adherent on basement membrane unless specifically induced to migrate at defined times (e.g., during tissue remodeling as a consequence of pregnancy and lactation) by changes in intracellular signalling pathways controlling the migratory apparatus of these cells; important components of this migratory apparatus include the $\alpha 3\beta 1$ integrin and the actin cytoskeleton. Early stages of malignant progression include constitutive

activation of these signalling pathways, which give rise to continually migrating cells. Later stages of malignant progression involve conversion from $\alpha 3\beta 1$ to a different $\beta 1$ integrin, and , perhaps activation of different signalling pathways. Our observation that more aggressive malignant cell lines use a different $\beta 1$ integrin(s) to bind and migrate on laminin-5 suggests that the conversion from static adhesion to migration through the $\alpha 3\beta 1$ integrin may therefore represent an early event in malignant progression, and as such, may provide a significant marker for early detection of breast cancer.

Our studies on antibody-stimulated migration of MCF-10A cells produced three remarkable findings. The first is that direct activation of the $\alpha 3\beta 1$ integrin with the monoclonal antibody TS2/16 alone induced migration in MCF-10A cells on laminin-5. TS2/16 has been used as an activating antibody of $\beta 1$ and $\beta 7$ integrin receptors in a number of cell types (80-84), and in virtually all cases this activation results in increased cell adhesion to integrin ligands such as fibronectin or members of the laminin family of ECM proteins. Inhibition of cell migration occurs concomitantly with increased adhesion in these cases. In contrast, we do not detect a significant increase in adhesion of integrin-activated MCF-10A cells to laminin-5 (not shown) and instead observe an increase in migration of these cells. Although $\alpha 3\beta 1$ supports migration of a variety of cells in response to soluble factors such as serum, growth factors, or cell conditioned media (85-88), this is the first report of direct integrin activation leading to cell migration through this receptor.

How $\alpha 3\beta 1$ integrin activation leads to cell migration is not clear. Despite the fact that integrins are widely accepted as activatable signalling receptors, the downstream consequences of integrin "activation," per se, are only loosely defined. Multiple downstream signalling molecules such as focal adhesion kinase, ion channels, members of the rho family of GTPases, and members of the mitogen activated protein (MAP) kinase pathway are stimulated by integrin clustering or activation (89-93). Our second significant observation, that TS2/16 activation of $\alpha 3\beta 1$ integrins appears to signal, at least in part, through the G_i class of heterotrimeric G proteins, adds another class of signalling proteins to this list. How these signalling proteins assemble into integrin-specific pathways is still not known, and is complicated by the fact that many of these proteins are used by multiple signalling pathways (e.g., growth factors, chemokines). While it is clear that there must be some specificity in the signals generated by these pathways, the use of common second messengers makes biochemical dissection of these pathways difficult at best.

The use of cAMP as a second messenger in TS2/16 stimulated cells is a case in point. In breast cells, cAMP is also used as a second messenger in signalling pathways stimulated by estrogen receptor, b-adrenergic receptors, prostaglandin E2, oxytocin, vasoactive intestinal peptide receptor, and calcitonin receptor (94-100). That TS2/16 stimulation induces a rise in cAMP, and antibody-induced migration is curbed by pertussis toxin and inhibitors of adenylate cyclase and PKA, strongly suggests that these proteins form a signalling pathway mediating migration in MCF-10A cells. This pathway must be functional for integrin-stimulated migration to take place, yet cAMP is clearly not a migration-specific second messenger, since other non-migratory stimuli also induce changes in cAMP levels.

We propose that the specificity of this pathway lies either upstream or downstream of

cAMP production. Our observation that antibody-induced migration is inhibited by pertussis toxin but not cholera toxin offers one possibility: integrin activation may act through the G_i class of G proteins, while other stimuli use G_s and other classes of G proteins to effect a rise in cAMP. Similarly, multiple isoforms of adenylate cyclase and protein kinase A exist, and they are differentially regulated by multiple signalling molecules (101-107), suggesting that a subset of these may mediate cell migration while others mediate the effects initiated by non-migratory stimuli such as adrenalin. This may explain why pharmacologic agents such as 8-br-cAMP and forskolin stimulate migration while adrenalin does not: cell migration may not simply depend on whether cAMP levels rise, but rather on *how* this rise is accomplished.

A third striking finding is that the cAMP/protein kinase A signalling pathway induces migration on laminin-5 but not laminin-1, suggesting that it exhibits at least some specificity at the ECM level. While laminin-5 has been shown to be a more potent stimulator of cell migration than laminin-1 in some cells, (108), this may at least be explained by the fact that normal and MCF-10A cells adhere to laminin-1 and laminin-5 via different integrins ($\alpha 6\beta 1$ and $\alpha 3\beta 1$, respectively; G.E. Plopper, unpublished), which in turn suggests that this signalling pathway may associate with only a subset of laminin binding integrin receptors.

In addition to supporting migration of MCF-10A cells via $\alpha 3\beta 1$ integrin, laminin-5 also supports branching morphogenesis and stable, static cell adhesion and formation of hemidesmosomes in these cells via the $\alpha 6\beta 4$ integrin (31). How these cells switch from a stationary to a migratory phenotype is unknown, and very little is known about the intracellular signalling activity associated with laminin-5 adhesion. To date only one report linking protein phosphorylation to $\alpha 6\beta 4$ -mediated adhesion to laminin-5 has appeared, and the significance of this finding to breast cell migration is unclear, since this phosphorylation has only been described in keratinocytes following deadhesion from laminin-5 (76). Our results suggest that the activation state of the $\alpha 3\beta 1$ integrin may play a central role in this conversion. How $\alpha 3\beta 1$ may be activated physiologically is not yet clear.

Finally, our observation that constitutive migration of malignant breast cells can be blocked by inhibitors of G proteins, adenylate cyclase, and protein kinase A suggests that these signalling proteins may play a role in the spread of malignant breast cancer. Our findings may also offer some functional significance for the observation that increased levels of cAMP binding proteins, including protein kinase A, are found in malignant breast tumors (109,110). Similarly, inhibition of these proteins may limit the migratory behavior of breast cells and thus decrease the spread of malignant breast cancer.

References

1. Barsky, S.H., Siegal, G.P., Jannotta, F., and Liotta, L.A. Loss of basement membrane components by invasive tumors but not by their benign counterparts. *Lab. Invest.*, **49**: 140-147, 1983.
2. Tsubura, A., Shikata, N., Inui, T., Morii, S., Hatano, T., Oikawa, T., and MatsuzawaA., Immunohistochemical localization of myoepithelial cells and basement membrane in normal, benign and malignant human breast lesions. *Virchows Archiv - A, Pathological Anatomy & Histopathology*, **413**: 133-139, 1988.
3. Kohn, E.C. and Liotta, L.A. Molecular insights into cancer invasion: strategies for prevention and intervention. *Cancer Res.*, **55**: 1856-1862, 1995.
4. Lauffenburger, D.A. and Horwitz, A.F. Cell migration: a physically integrated molecular process. [Review]. *Cell*, **84**: 359-369, 1996.
5. Stossel, T.P. On the crawling of animal cells. *Science*, **260**: 1086-1094, 1993.
6. Guan, J.L. and Chen, H.C. Signal transduction in cell-matrix interactions. [Review] [244 refs]. *Int. Rev. Cytol.*, **168**: 81-121, 1996.
7. Liotta, L.A., Steeg, P.S., and Stetler-Stevenson, W.G. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. *Cell*, **64**: 327-336, 1991.
8. Magro, G., Lanzafame, S., and Colombatti, A. Immunohistochemical staining patterns of type VI collagen in the normal, hyperplastic, and neoplastic adult male breast. *Pathologica*, **86**: 142-145, 1994.
9. Christensen, L. The distribution of fibronectin, laminin and tetranectin in human breast cancer with special attention to the extracellular matrix. [Review]. *Apmis, Supplementum*. **26**: 1-39, 1992.
10. Coopman, P., Verhasselt, B., Bracke, M., De Bruyne, G., Castronovo, V., Sobel, M., Foidart, J.M., Van Roy, F., and Mareel, M. Arrest of MCF-7 cell migration by laminin in vitro: possible mechanisms. *Clin. Exp. Metastasis*, **9**: 469-484, 1991.
11. D'Ardenne, A.J., Richman, P.I., Horton, M.A., McAulay, A.E., and Jordan, S. Coordinate expression of the alpha-6 integrin laminin receptor sub-unit and laminin in breast cancer. *J. Pathol.*, **165**: 213-220, 1991.
12. Yoshida, T., Ishihara, A., Hirokawa, Y., Kusakabe, M., and Sakakura, T. Tenascin in breast cancer development--is epithelial tenascin a marker for poor prognosis? [Review]. *Cancer Lett.*, **90**: 65-73, 1995.
13. Jaskiewicz, K., Chasen, M.R., and Robson, S.C. Differential expression of extracellular matrix proteins and integrins in hepatocellular carcinoma and chronic liver disease. *Anticancer Res.*, **13**: 2229-2237, 1993.

14. Shoji, T., Kamiya, T., Tsubura, A., Hamada, Y., Hatano, T., Hioki, K., and Morii, S. Tenascin staining positivity and the survival of patients with invasive breast carcinoma. *J. Surg. Res.*, **55**: 295-297, 1993.
15. Moch, H., Torhorst, J., Durmuller, U., Feichter, G.E., Sauter, G., and Gudat, F. Comparative analysis of the expression of tenascin and established prognostic factors in human breast cancer. *Pathol. Res. Pract.*, **189**: 510-514, 1993.
16. Narita, T., Kawakami-Kimura, N., Sato, M., Matsuura, N., Higashiyama, S., and Kannagi, R. Alteration of integrins by heparin-binding EGF-like growth factor in human breast cancer cells. *Oncology*, **53**: 374-381, 1996.
17. Gui, G.P., Wells, C.A., Yeomans, P., Jordan, S.E., Vinson, G.P., and Carpenter, R. Integrin expression in breast cancer cytology: a novel predictor of axillary metastasis. *European Journal of Surgical Oncology*, **22**: 254-258, 1996.
18. Tawil, N.J., Gowri, V., Djoneidi, M., Nip, J., Carbonetto, S., and Brodt, P. Integrin alpha3beta1 can promote adhesion and spreading of metastatic breast carcinoma cells on the lymph node stroma. *Int. J. Cancer*, **66**: 703-710, 1996.
19. Shaw, L.M., Chao, C., Wewer, U.M., and Mercurio, A.M. Function of the integrin alpha 6 beta 1 in metastatic breast carcinoma cells assessed by expression of a dominant-negative receptor. *Cancer Res.*, **56**: 959-963, 1996.
20. Maemura, M., Akiyama, S.K., Woods, V.L., Jr., and Dickson, R.B. Expression and ligand binding of alpha 2 beta 1 integrin on breast carcinoma cells. *Clin. Exp. Metastasis*, **13**: 223-235, 1995.
21. Glukhova, M., Koteliansky, V., Sastre, X., and Thiery, J.P. Adhesion systems in normal breast and in invasive breast carcinoma. *Am. J. Pathol.*, **146**: 706-716, 1995.
22. Friedrichs, K., Ruiz, P., Franke, F., Gille, I., Terpe, H.J., and Imhof, B.A. High expression level of alpha 6 integrin in human breast carcinoma is correlated with reduced survival. *Cancer Res.*, **55**: 901-906, 1995.
23. Gui, G.P., Wells, C.A., Browne, P.D., Yeomans, P., Jordan, S., Puddefoot, J.R., and Carpenter, R. Integrin expression in primary breast cancer and its relation to axillary nodal status. *Surgery*, **117**: 102-108, 1995.
24. Oda, K., Itoh, H., Utsunomiya, H., Itoh, J., Osamura, R.Y., Tokuda, Y., and Kubota, M. Bio-histochemical aspects of integrins (alpha 2 beta 1, alpha 6 beta 1) in invasive mammary carcinomas: an immunohistochemical study. *Pathol Int.*, **44**: 435-441, 1994.
25. Arihiro, K., Inai, K., Kurihara, K., Takeda, S., Khatun, N., Kuroi, K., and Toge, T. A role of VLA-6 laminin receptor in invasion of breast carcinoma. *Acta Pathol. Jpn.*, **43**: 662-669, 1993.
26. Sager, R., Anisowicz, A., Neveu, M., Liang, P., and Sotiropoulou, G. Identification

by differential display of alpha 6 integrin as a candidate tumor suppressor gene. FASEB J., 7: 964-970, 1993.

27. Jones, J.L., Critchley, D.R., and Walker, R.A. Alteration of stromal protein and integrin expression in breast--a marker of premalignant change. *J. Pathol.*, 167: 399-406, 1992.

28. Natali, P.G., Nicotra, M.R., Botti, C., Mottolese, M., Bigotti, A., and Segatto, O. Changes in expression of alpha 6/beta 4 integrin heterodimer in primary and metastatic breast cancer. *Br. J. Cancer*, 66: 318-322, 1992.

29. Koukoulis, G.K., Virtanen, I., Korhonen, M., Laitinen, L., Quaranta, V., and GouldVE., Immunohistochemical localization of integrins in the normal, hyperplastic, and neoplastic breast. Correlations with their functions as receptors and cell adhesion molecules. *Am. J. Pathol.*, 139: 787-799, 1991.

30. Hewitt, R.E., Powe, D.G., Morrell, K., Balley, E., Leach, I.H., Ellis, I.O., and TurnerDR., Laminin and collagen IV subunit distribution in normal and neoplastic tissues of colorectum and breast. *Br. J. Cancer*, 75: 221-229, 1997.

31. Stahl, S., Weitzman, S., and Jones, J.C.R. The role of laminin-5 and its receptors in mammary epithelial cell branching morphogenesis. *J. Cell Sci.*, 110: 55-63, 1997.

32. Pyke, C., Salo, S., Ralfkiaer, E., Romer, J., Dano, K., and Tryggvason, K. Laminin-5 is a marker of invading cancer cells in some human carcinomas and is coexpressed with the receptor for urokinase plasminogen activator in budding cancer cells in colon adenocarcinomas. *Cancer Res.*, 55: 4132-4139, 1995.

33. Schwartz, M.A., Schaller, M.D., and Ginsberg, M.H. Integrins: Emerging Paradigms of Signal Transduction. *Annu. Rev. Cell Dev. Biol.*, 11: 549-599, 1995.

34. D'Souza, B., Berdichevsky, F., Kyprianou, N., and Taylor-Papadimitriou, J. Collagen-induced morphogenesis and expression of the alpha 2-integrin subunit is inhibited in c-erbB2-transfected human mammary epithelial cells. *Oncogene*, 8: 1797-1806, 1993.

35. Gordge, P.C., Hulme, M.J., Clegg, R.A., and Miller, W.R. Elevation of protein kinase A and protein kinase C activities in malignant as compared with normal human breast tissue. *Eur. J. Cancer*, 32A: 2120-2126, 1996.

36. Hawkins, R.A., Tesdale, A.L., Killen, M.E., Jack, W.J., Chetty, U., Dixon, J.M., Prescott, R.J., McIntyre, M.A., and Miller, W.R. Prospective evaluation of prognostic factors in operable breast cancer. *Br. J. Cancer*, 74: 1469-1478, 1996.

37. Carley, W.W., Lipsky, M.G., and Webb, W.W. Regulation and drug insensitivity of F-actin association with adhesion areas of transformed cells. *J. Cell. Physiol.*, 117: 257-265, 1983.

38. Cary, L.A., Chang, J.F., and Guan, J.L. Stimulation of cell migration by

overexpression of focal adhesion kinase and its association with Src and Fyn. *J. Cell Sci.*, **109**: 1787-1794, 1996.

39. Illc, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., NomuraS., Fujimoto, J., Okada, M., Yamamoto, T., and et al., Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature*, **377**: 539-544, 1995.

40. Ylanne, J. and Virtanen, I. The Mr 140,000 fibronectin receptor complex in normal and virus-transformed human fibroblasts and in fibrosarcoma cells: identical localization and function. *Int. J. Cancer*, **43**: 1126-1136, 1989.

41. Guan, J.L. and Shalloway, D. Regulation of focal adhesion-associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation. *Nature*, **358**: 690-692, 1992.

42. Schaller, M.D., Borgman, C.A., Cobb, B.S., Vines, R.R., Reynolds, A.B., and Parsons, J.T. pp125FAK a structurally distinctive protein-tyrosine kinase associated with focal adhesions. *Proc. Natl. Acad. Sci. U. S. A.*, **89**: 5192-5196, 1992.

43. Dhanasekaran, N., Heasley, L.E., and Johnson, G.L. G protein-coupled receptor systems involved in cell growth and oncogenesis. *Endocrine Rev.*, **16**: 259-270, 1995.

44. Burnette, W.N. AB5 ADP-ribosylating toxins: comparative anatomy and physiology. *Structure*, **2**: 151-158, 1994.

45. Hauzenberger, D., Klominek, J., and Sundqvist, K. Functional specialization of fibronectin-binding beta-1- integrins in T lymphocyte migration. *J. Immunol.*, **153**: 960-970, 1994.

46. Aznavoorian, S., Strake, M.L., Krutsch, H., Schiffman, E., and Liotta, L.A. Signal transduction for chemotaxis and haptotaxis by matrix molecules in tumor cells. *J. Cell Biol.*, **110**: 1427-1438, 1990.

47. Band, V. and Sager, R. Distinctive traits of normal and tumor-derived human mammary epithelial cells expressed in a medium that supports long-term growth of both cell types. *Proc. Natl. Acad. Sci. U. S. A.*, **86**: 1249-1253, 1989.

48. Plopper, G., Falk-Marzillier, J., Glaser, S., Fitchmun, M., Giannelli, G., Romano, T., Jones, J.C.R., and Quaranta, V. Changes in expression of monoclonal antibody epitopes on laminin-5r induced by cell contact. *J. Cell Sci.*, **109**: 1965-1973, 1996.

49. Fradet, Y., Cordon-Cardo, C., Thomson, T., Daly, M.E., and Whitmore, W.F. Cell surface antigens of human bladder cancer defined by mouse monoclonal antibodies. !!!BADKEY -- Journal Name Lost, 1984.

50. Lehmann, M., Rabenandrasana, C., Tamura, R.N., Lissitzky, J-C, Quaranta, V., Pinchon, J., and Marvaldi, J. A monoclonal antibody inhibits adhesion to fibronectin and vitronectin of a colon carcinoma cell line and recognizes the integrins avb3, avb5

and avb6. *Cancer Res.*, **54**: 2102-2107, 1994.

51. Gospodarowicz, D. Preparation of Extracellular Matrices Produced by Cultured Bovine Corneal Endothelial Cells and PF-HR-9 Endodermal Cells: Their Use in Cell Culture. In: D.W. Barnes, D.A. Sirbasku and G.H. Stao (eds.), *Methods for Preparation of Media, Supplements and Substrata*, pp. 275-293, New York: Alan R. Liss, Inc.. 1984.
52. Albrecht-Buehler, G. The phagokinetic tracks of 3T3 cells. *Cell*, **11**: 395-404, 1977.
53. Langhofer, M., Hopkinson, S.B., and Jones, J.C.R. The matrix secreted by 804G cells contains laminin related components that participate in hemidesmosome assembly in vitro. *J. Cell Sci.*, **105**: 753-764, 1993.
54. Tamura, R.N., Cooper, H.M., Collo, G., and Quaranta, V. Cell-type specific integrin variants with alternative a chain cytoplasmic domains. *Proc. Natl. Acad. Sci. U. S. A.*, **88**: 10183-10187, 1991.
55. Takada, Y., Murphy, E., Pil, P., Chen, C., Ginsberg, M.H., and Hemler, M.E. Molecular cloning and expression of the cDNA for alpha3 subunit of human alpha3 beta1 (VLA-3), an integrin receptor for fibronectin laminin and collagen. *J. Cell Biol.*, **115**: 257-266, 1991.
56. Soule, H.D., Maloney, T.M., Wolman, S.R., Peterson, W.D., Jr., Brenz, R., McGrath, C.M.R., Pauley, R.J., Jones, R.F., and Brooks, S.C. Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. *Cancer Res.*, **50**: 6075-6086, 1990.
57. Gelmann, E.P., Thompson, E.W., and Sommers, C.L. Invasive and metastatic properties of MCF-7 cells and rasH-transfected MCF-7 cell lines. *Int. J. Cancer*, **50**: 665-669, 1992.
58. Zhang, R.D., Fidler, I.J., and Price, J.E. Relative malignant potential of human breast carcinoma cell lines established from pleural effusions and a brain metastasis. *Invasion Metastasis*, **11**: 204-215, 1991.
59. Price, J.E., Polyzos, A., Zhang, R.D., and Daniels, L.M. Tumorigenicity and metastasis of human breast carcinoma cell lines in nude mice. *Cancer Res.*, **50**: 717-721, 1990.
60. Wayner, E.A., Carter, W.G., Piotrowicz, R.S., and Kunicki, T.J. The function of multiple extracellular matrix receptors in mediating cell adhesion to extracellular matrix receptors: Preparation of monoclonal antibodies to the fibronectin receptor that specifically inhibit cell adhesion to fibronectin and react with platelet glycoproteins Ic-IIa. *J. Cell Biol.*, **107**: 1881-1891, 1989.
61. Sonnenberg, A., Janssen, H., Hogervorst, F., Calafat, J., and Hilgers, J. A complex of platelet glycoproteins Ic and IIa identified by a rat monoclonal antibody. *J. Biol. Chem.*, **262**: 10376-10383, 1987.

62. Carter, W.G., Wayner, E.A., Bouchard, T.S., and Kaur, P. The role of integrins alpha 2 beta 1 and alpha 3 beta 1 in cell-cell and cell-substrate adhesion of human epidermal cells. *J. Cell Biol.*, 110: 1387-1404, 1990.

63. Baker, S.E., Hopkinson, S.B., Fitchmun, M., Andreason, G.L., Frasier, F., Plopper, G., Quaranta, V., and Jones, J.C.R. Laminin-5 and hemidesmosomes: role of the a3 chain subunit in hemidesmosome stability and assembly. *J. Cell Sci.*, 109: 2509-2520, 1996.

64. Bassler, R. and Katzer, B. Histopathology of myoepithelial (basocellular) hyperplasias in adenosis and epitheliosis of the breast demonstrated by the reactivity of cytokeratins and S100 protein. An analysis of heterogenic cell proliferations in 90 cases of benign and malignant breast diseases. *Virchows Archiv - A, Pathological Anatomy & Histopathology*, 421: 435-442, 1992.

65. Raymond, W.A. and Leong, A.S. Assessment of invasion in breast lesions using antibodies to basement membrane components and myoepithelial cells. *Pathology*, 23: 291-297, 1991.

66. Leathem, A.J. Biological, biochemical and morphological markers of breast disorders and of breast cancer. [Review] [41 refs]. *Acta Histochemica - Supplementband*, 40: 51-58, 1990.

67. Curschellas, E., Matter, A., and Regenass, U. Immunolocalization of cytoskeletal elements in human mammary epithelial cells. *European Journal of Cancer & Clinical Oncology*, 23: 1517-1527, 1987.

68. Badley, R.A., Couchman, J.R., and Rees, D.A. Comparison of the cell cytoskeleton in migratory and stationary chick fibroblasts. *Journal of Muscle Research & Cell Motility*, 1: 5-14, 1980.

69. Mitchison, T.J. and Cramer, L.P. Actin-Based Cell Motility and Cell Locomotion. *Cell*, 84: 371-379, 1996.

70. Pellegrini, R., Martignone, S., Tagliabue, E., Belotti, D., Bufalino, R., Menard, S., and Colnaghi, M.I. Prognostic significance of laminin production in relation with its receptor expression in human breast carcinomas. *Breast Cancer Res. Treat.*, 35: 195-199, 1995.

71. Soini, Y., Hurskainen, T., Hoyhtya, M., Oikarinen, A., and Autio-Harmainen, H. 72 KD and 92 KD type IV collagenase, type IV collagen, and laminin mRNAs in breast cancer: a study by in situ hybridization. *J. Histochem. Cytochem.*, 42: 945-951, 1994.

72. Gorczyca, W., Holm, R., and Nesland, J.M. Laminin production and fibronectin immunoreactivity in breast carcinomas. *Anticancer Res.*, 13: 851-858, 1993.

73. Lelievre, S., Weaver, V.M., and Bissell, M.J. Extracellular matrix signaling from the cellular membrane skeleton to the nuclear skeleton: a model of gene regulation.

[Review]. Recent Progress in Hormone Research, 51: 417-432, 1996.

74. Niessen, C.M., Hogervorst, F., Jaspars, L.H., de Melker, A.A., Delwel, G.O., Kuikman, I., and Sonnenberg, A. The alpha 6 beta 4 integrin is a receptor for both laminin and kalinin. *Exp. Cell Res.*, 211: 360-367, 1994.

75. Anbazhagan, R., Bartek, J., Stamp, G., Pignatelli, M., and Gusterson, B. Expression of integrin subunits in the human infant breast correlates with morphogenesis and differentiation. *J. Pathol.*, 176: 227-232, 1995.

76. Xia, Y., Gil, S.G., and Carter, W.G. Anchorage mediated by integrin alpha₆beta₄ to laminin 5 (epiligrin) regulates tyrosine phosphorylation of a membrane-associated 80-kD protein. *J. Cell Biol.*, 132: 727-740, 1996.

77. Pyke, C., Romer, J., Kallunki, P., Lund, L.R., Ralfkiaer, E., Dano, K., and Tryggvason, K. The gamma 2 chain of kalinin/laminin 5 is preferentially expressed in invading malignant cells in human cancers. *Am. J. Pathol.*, 145: 782-791, 1994.

78. Rousselle, P., Lunstrum, G.P., Keene, D.R., and Burgeson, R.E. Kalinin: an epithelium-specific basement membrane adhesion molecule that is a component of anchoring filaments. *J. Cell Biol.*, 114: 567-576, 1991.

79. Tawil, N., Wilson, P., and Carbonetto, S. Integrins in point contacts mediate cell spreading: Factors that regulate integrin accumulation in point contacts vs. focal contacts. *J. Cell Biol.*, 120: 261-271, 1993.

80. Dominguez-Jimenez, C., Sanchez-Aparicio, P., Albar, J.P., and Garcia-Pardo, A. The alpha 4 beta 1 fibronectin ligands CS-1, Hep II, and RGD induce different intracellular events in B lymphoid cells. Comparison with the effects of the endothelial ligand VCAM-1. *Cell Adhes. Commun.*, 4: 251-267, 1996.

81. Walsh, G.M., Symon, F.A., Lazarovits, A.L., and Wardlaw, A.J. Integrin alpha 4 beta 7 mediates human eosinophil interaction with MAdCAM-1, VCAM-1 and fibronectin. *Immunology*, 89: 112-119, 1996.

82. Weber, C., Alon, R., Moser, B., and Springer, T.A. Sequential regulation of alpha 4 beta 1 and alpha 5 beta 1 integrin avidity by CC chemokines in monocytes: implications for transendothelial chemotaxis. *J. Cell Biol.*, 134: 1063-1073, 1996.

83. Luque, A., Sanchez-Madrid, F., and Cabanas, C. Functional regulation of the human integrin VLA-1 (CD49a/CD29) by divalent cations and stimulatory beta 1 antibodies. *FEBS Lett.*, 346: 278-284, 1994.

84. Delwel, G.O., de Melker, A.A., Hogervorst, F., Jaspars, L.H., Fles, D.L., Kuikman, I., Lindblom, A., Paulsson, M., Timpl, R., and Sonnenberg, A. Distinct and overlapping ligand specificities of the alpha 3A beta 1 and alpha 6A beta 1 integrins: recognition of laminin isoforms. *Mol. Biol. Cell*, 5: 203-215, 1994.

85. Herard, A.L., Pierrot, D., Hinnrasky, J., Kaplan, H., Sheppard, D., and Puchelle, E.

Fibronectin and its alpha 5 beta 1-integrin receptor are involved in the wound-repair process of airway epithelium. Am. J. Physiol., 271: L726-L733, 1996.

86. Zhang, K. and Kramer, R.H. Laminin 5 deposition promotes keratinocyte motility. Exp. Cell Res., 227: 309-322, 1996.

87. Melchiori, A., Mortarini, R., Carbone, S., Marchisio, P.C., Anichini, A., Noonan DM., and Albini, A. The alpha 3 beta 1 integrin is involved in melanoma cell migration and invasion. Exp. Cell Res., 219: 233-242, 1995.

88. Okada, T., Okuno, H., and Mitsui, Y. A novel in vitro assay system for transendothelial tumor cell invasion: significance of E-selectin and alpha 3 integrin in the transendothelial invasion by HT1080 fibrosarcoma cells. Clin. Exp. Metastasis, 12: 305-314, 1994.

89. Schwartz, M.A., Schaller, M.A., and Ginsberg, M.H. Integrins: Emerging paradigms of signal transduction. Annu. Rev. Cell Dev. Biol., 11: 549-599, 1995.

90. Clark, E.A. and Brugge, J.S. Integrins and signal transduction pathways: the road taken. [Review]. Science, 268: 233-239, 1995.

91. Hotchin, N.A. and Hall, A. Regulation of the actin cytoskeleton, integrins and cell growth by the Rho family of small GTPases. [Review] [73 refs]. Cancer Surveys, 27: 311-322, 1996.

92. Lafrenie, R.M. and Yamada, K.M. Integrin-dependent signal transduction. [Review] [69 refs]. Journal of Cellular Biochemistry, 61: 543-553, 1996.

93. Parsons, J.T. Integrin-mediated signalling: regulation by protein tyrosine kinases and small GTP-binding proteins. [Review] [47 refs]. Curr. Opin. Cell Biol., 8: 146-152, 1996.

94. el-Tanani, M.K. and Green, C.D. Interaction between estradiol and cAMP in the regulation of specific gene expression. Molecular & Cellular Endocrinology, 124: 71-77, 1996.

95. Badino, G.R., Novelli, A., Girardi, C., and Di Carlo, F. Evidence for functional beta-adrenoceptor subtypes in CG-5 breast cancer cell. Pharmacological Research, 33: 255-260, 1996.

96. Zhao, Y., Agarwal, V.R., Mendelson, C.R., and Simpson, E.R. Estrogen biosynthesis proximal to a breast tumor is stimulated by PGE2 via cyclic AMP, leading to activation of promoter II of the CYP19 (aromatase) gene. Endocrinology, 137: 5739-5742, 1996.

97. Zingg, H.H. Vasopressin and oxytocin receptors. [Review] [118 refs]. Baillieres Clinical Endocrinology & Metabolism, 10: 75-96, 1996.

98. Zia, H., Hida, T., Jakowlew, S., Birrer, M., Gozes, Y., Reubi, J.C., Fridkin, M., and

Moody, T.W. Breast cancer growth is inhibited by vasoactive intestinal peptide (VIP) hybrid, a synthetic VIP receptor antagonist. *Cancer Res.*, **56**: 3486-3489, 1996.

99. Albrandt, K., Brady, E.M., Moore, C.X., Mull, E., Sierzega, M.E., and Beaumont, K. Molecular cloning and functional expression of a third isoform of the human calcitonin receptor and partial characterization of the calcitonin receptor gene. *Endocrinology*, **136**: 5377-5384, 1995.

100. Aronica, S.M., Kraus, W.L., and Katzenellenbogen, B.S. Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription. *Proc. Natl. Acad. Sci. U. S. A.*, **91**: 8517-8521, 1994.

101. Iyengar, R. Multiple families of Gs-regulated adenylyl cyclases. [Review] [31 refs]. *Advances in Second Messenger & Phosphoprotein Research*, **28**: 27-36, 1993.

102. Pieroni, J.P., Jacobowitz, O., Chen, J., and Iyengar, R. Signal recognition and integration by Gs-stimulated adenylyl cyclases. [Review] [42 refs]. *Current Opinion in Neurobiology*, **3**: 345-351, 1993.

103. Choi, E.J., Xia, Z., Villacres, E.C., and Storm, D.R. The regulatory diversity of the mammalian adenylyl cyclases. [Review] [28 refs]. *Curr. Opin. Cell Biol.*, **5**: 269-273, 1993.

104. Beebe, S.J. The cAMP-dependent protein kinases and cAMP signal transduction. [Review] [73 refs]. *Semin. Cancer Biol.*, **5**: 285-294, 1994.

105. Walsh, D.A. and Van Patten, S.M. Multiple pathway signal transduction by the cAMP-dependent protein kinase. [Review] [45 refs]. *FASEB J.*, **8**: 1227-1236, 1994.

106. Coghlan, V.M., Bergeson, S.E., Langeberg, L., Nilaver, G., and Scott, J.D. A-kinase anchoring proteins: a key to selective activation of cAMP-responsive events?. [Review] [103 refs]. *Mol. Cell. Biochem.*, **127-128**: 309-319, 1993.

107. Meinkoth, J.L., Alberts, A.S., Went, W., Fantozzi, D., Taylor, S.S., Hagiwara, M., and Feramisco, J.R. Signal transduction through the cAMP-dependent protein kinase. [Review] [35 refs]. *Mol. Cell. Biochem.*, **127-128**: 179-186, 1993.

108. Kikkawa, Y., Umeda, M., and Miyazaki, K. Marked stimulation of cell adhesion and motility by ladsin, a laminin-like scatter factor. *J. Biochem.*, **116**: 862-869, 1994.

109. Miller, W.R., Watson, D.M., Jack, W., Chetty, U., and Elton, R.A. Tumour cyclic AMP binding proteins: an independent prognostic factor for disease recurrence and survival in breast cancer. *Breast Cancer Res. Treat.*, **26**: 89-94, 1993.

110. Miller, W.R., Hulme, M.J., Cho-Chung, Y.S., and Elton, R.A. Types of cyclic AMP binding proteins in human breast cancers. *Eur. J. Cancer*, **29A**: 989-991, 1993.

Cell Line	P1E6 ($\alpha 2$)	P1B5 ($\alpha 3$)	GoH3 ($\alpha 6$)	P4C10 ($\beta 1$)	Anti-Mouse 2°	Anti-Rat 2°
HUMEC	19.02	9.96	16.4	9.59	3.49	3.36
MCF-10A	23.45	21.54	26.52	17.64	2.37	2.13
MCF-7	18.28	22.29	20.21	25.4	4.82	4.81
MDA-MB-435	19.21	20.08	36.09	13.76	3.44	3.54
MDA-MB-231	17.05	21.65	36.29	19.92	3.61	3.65

Table 1. Mammary epithelial cells express $\alpha 2$, $\alpha 3$, $\alpha 6$, and $\beta 1$ integrins. HUMEC, MCF-10A, MCF-7, MDA-MB-231, and MDA-MB-435 cells were stained with the monoclonal antibodies directed against the following integrin subunits: P1E6 = $\alpha 2$, P1B5 = $\alpha 3$, GoH3 = $\alpha 6$, P4C10 = $\beta 1$, followed by secondary antibodies conjugated to fluorescein, and analyzed by flow cytometry. As controls, cells were also stained with secondary antibodies alone. Note that all cell types expressed all four integrins tested. Results are expressed as mean fluorescence intensity (log scale, arbitrary units) of 10,000 sorted cells.

	HUMEC	MCF-10A	MCF-7	MDA-MB-231	MDA-MB-435
Continual Growth in Culture	-	+	+	+	+
Preferential Adhesion to Laminin-5	+	-	-	-	-
Constitutive, Random Migration on Laminin-5	-	n/d	+	+	+
Constitutive, Haptotactic Migration on Laminin-5	-	-	+	+	+
$\alpha\beta 1$ as Laminin-5 Receptor	+	+	+	-	-

Table 2. In vitro growth, adhesion, and migration behavior of primary (HUMEC), immortalized (MCF-10A), and three malignant breast epithelial cells that exhibit different degrees of metastatic potential (MDA-MB-435>MDA-MB-231>MCF-7). This table illustrates the following three trends: 1) Continuously proliferating cells lose preferential adhesion to laminin-5; 2) Malignant cells are constitutively migratory on laminin-5; 3) The most malignant cells abandon $\alpha\beta 1$ integrin as the adhesive and migratory receptor for laminin-5.

Figure Legends

Figure 1. laminin-5 is expressed in basal membranes of the breast epithelium. Panels A, B, and C show representative fields of a frozen section immunostained with anti-laminin-5 monoclonal antibody CM6, and observed with a confocal system. Note the clear and sharp immunofluorescent staining detected at the basal (basolateral) pole of breast epithelial cells by this mAb. Under the same confocal settings, no detectable signal was recorded in sections incubated with control isotype matched mouse IgGs (panel D). Images in panels A, B, and D were collected by a 40X (1.3 NA) objective, at a zoom factor 1, whereas the image in panel C was obtained at a zoom factor 3.

Figure 2. Normal breast epithelial cells preferentially bind laminin-5. Normal (HUMEC), immortalized (MCF-10A), and malignant cells were plated on the indicated extracellular matrix molecules for 30 minutes, gently washed to remove unattached cells, then fixed, stained and quantitated as described in Materials and Methods. Ln-5 = laminin-5; Ln-2 = human laminin-2; Fn = human fibronectin; Ln-1 = rat laminin-1. Data are presented as the statistical mean +/- standard deviation (n=8).

Figure 3. Inhibition of breast cell adhesion to laminin-5 by anti-integrin antibodies. Cells were plated on laminin-5 for 30 minutes in the presence of antibodies directed against the following integrin subunits: P1E6 = α 2, P1B5 = α 3, GoH3 = α 6, P4C10 = β 1. Cells were then gently washed, fixed, stained, and quantitated as for Fig. 2. Note that adhesion of HUMEC, MCF-10A, and MCF-7 cell lines was completely blocked by antibodies against α 3 and β 1. Ln-5 = laminin-5.

Figure 4. HUMEC and MCF-7 cells express identical isoforms of the α 3 integrin. (A). Expression of the α 3A isoform was detected by RT-PCR of mRNA isolated from HUMEC and MCF-7 cells. Primers flanking the sequences encoding the alternatively spliced cytoplasmic domains amplified a 570 bp band corresponding to α 3A, but no 426 bp band corresponding to α 3B. (B). Immunoprecipitation of α 3 subunits from cell surface, biotin-labeled HUMEC and MCF-7 cells. α 3 subunits from both cell types migrate at approximately 130 kDa. Migration of molecular weight standards is indicated at left.

Figure 5. The monoclonal antibody CM6 blocks adhesion of breast epithelial cells to laminin-5. Cells were plated for 30 minutes on plates coated with laminin-5 affinity captured from 804G cell conditioned medium by the indicated antibodies. Note that no cells adhered to wells coated with CM6. As controls, wells were coated with the anti-laminin-5 antibody TR1, irrelevant mouse IgG₁ (MOPC), or no antibody. Results are expressed as in Fig. 2.

Figure 6. MCF-7 cells migrate approximately 10-fold more than HUMECs on laminin-5 coated colloidal gold. A. Dark field images of indicated cells plated on colloidal gold particles coated with laminin-5 (Ln-5) or blotto (-CTL). Bar = 40 μ m. B. Quantitation of cell migration. Photographs of dark field microscopic fields as shown in panel A were digitized and scanned for black areas where cells had migrated. Values are expressed as the mean percentage of black area per field \pm standard deviation (n=3).

Figure 7. MCF-10A cells form mature focal adhesions and elaborate actin bundles

following adhesion to laminin-5. MCF-10A (a, c, e) and MCF-7 (b, d, f) cells were plated for 90 minutes on glass coverslips coated with laminin-5, then fixed, permeabilized, and stained with rhodamine-conjugated phalloidin to reveal filamentous actin (a, b), anti-vinculin antibody (c, d) or anti- α 3 integrin antibody. Bar = 20 μ m.

Figure 8. Inhibition of breast cell migration on laminin-5 by inhibitory antibodies. Mammary epithelial cells were plated on Transwell filters whose reverse sides were coated with bovine serum albumin (BSA) or laminin-5, in the presence of either CM6 or antibodies directed against the indicated integrin subunits: P1E6 = α 2, P1B5 = α 3, GoH3 = α 6, P4C10 = β 1. No inhibitory antibodies were included in control wells. After 18 hours, cells were fixed and stained, and migration quantitated as described in Materials and Methods. Results are expressed as statistical mean of number of cells counted per microscopic field (300x magnification) \pm standard deviation (n=8).

Figure 9. Integrin activation stimulates MCF-10A cell migration on laminin-5. Cells suspended in the indicated media components were placed in the upper chamber of Transwell filters whose undersides were coated with laminin-5 and allowed to migrate through the filter for 18 hours. Cells were then fixed, stained, and migrated cells counted as described in Materials and Methods. MM = minimal medium; DFCI = complete cell growth medium; TS2/16 = 50 μ g/ml integrin stimulating antibody unless otherwise indicated; P1B5 = 1:50 dilution of α 3 integrin blocking antibody ascites fluid; P5D2 = 50 μ g/ml non-stimulating β 1 antibody; BPE = bovine pituitary extract [40 μ g/ml]; EGF = recombinant human epidermal growth factor [12.5 nM]; Insulin = 1 μ g/ml purified bovine insulin. Note that cells do not migrate in MM, but are stimulated to migrate by all additives to this medium except non-stimulating β 1 antibody, and that TS2/16-stimulated migration is inhibited by anti- α 3 integrin antibody. Results are expressed as the mean of eight randomly selected fields on two separate filters per condition, \pm standard deviation.

Figure 10. TS2/16 does not stimulate MCF-10A cell migration on laminin-1. Cells were plated in the indicated media components on filters whose undersides were coated with 40 μ g/ml laminin-1 and processed for migration as described in Figure 1. Note that while complete growth medium (DFCI) stimulated cell migration, TS2/16 did not.

Figure 11. TS2/16 stimulates MCF-10A cell migration via a pertussis toxin-sensitive signalling pathway. MCF-10A cells suspended in the indicated media components were placed in Transwell migration assay chambers coated with laminin-5. Note that TS2/16 stimulated migration was inhibited by 100 ng/ml pertussis toxin (PTX), but not by 100 ng/ml cholera toxin (CTX). As controls, cells were also plated in MM alone, MM + 1% serum, or in DFCI medium \pm PTX or CTX. Results expressed as in Figure 1.

Figure 12. TS2/16-stimulated cells migrate on laminin-5 via a cAMP signalling pathway. MCF-10A cells incubated in MM supplemented with the indicated compounds were plated in Transwell migration assay chambers coated with laminin-5 or laminin-1 and processed for cell migration as described in Figure 8. Note that dibutyryl cAMP (dbcAMP, 500 nM), 8-bromo-cAMP (8-br-cAMP, 500 nM), and forskolin (FSK, 5.0 nM) stimulate MCF-10A cell migration on laminin-5 but not on laminin-1, and that this

stimulation is not additive with TS2/16 stimulation. Note also that inhibition of adenylate cyclase with SQ22536 (250 mM) or of protein kinase A with H-89 (4 mM) blocked TS2/16 stimulated migration. As controls, cells were plated in MM alone or in DFCI medium. Results expressed as in Figure 8.

Figure 13. Integrin clustering with anti-integrin antibody TS2/16 stimulates a rise in intracellular cAMP in MCF-10A cells. Purified TS2/16 (50 µg/ml) was added to 1×10^6 suspended cells in minimal migration medium for 30 minutes. Cells were lysed and cAMP collected from supernatants of acid precipitated protein. cAMP was quantitated by elisa assay and is expressed as fmol per mg of protein per sample. As controls, cells were treated with additional anti-integrin antibodies (P4C10, P5D2) with or without a crosslinking secondary anti-mouse Fc antibody (2° Ab) as well as compounds that activate cAMP signalling in other cell types (bombesin, bradykinin, adrenalin) or left untreated (control).

Figure 14. Migration of malignant breast cells is blocked by inhibitors of G proteins, adenylate cyclase, and protein kinase A. Malignant cells were added to Transwell migration assays containing laminin-5 as a substrate, in the presence or absence of inhibitors used in Figure 11. Note that migration of all three cell lines is inhibited by cholera toxin as well as those inhibitors that block TS2/16 stimulated migration in MCF-10A cells. Results expressed as in Figure 8.

Bibliography of publications and meeting abstracts:

1. Plopper, G., J. Falk-Marzillier, S. Glaser, M. Fitchmun, T. Romano, G. Gianelli, J.C.R. Jones, V. Quaranta. Changes in expression of monoclonal antibody epitopes on laminin-5r induced by cell contact. *J. Cell Sci.* 109: 1965-1973, 1996.
2. Baker, S.E., Hopkinson, S.B., Fitchmun, M., Andreason, G.L., Frasier, F., Plopper, G., Quaranta, V. and Jones, J.C.R. Laminin-5 and hemidesmosomes: role of the $\alpha 3$ chain subunit in hemidesmosome stability and assembly *J. Cell Sci.* 109: 2509 - 2520, 1996.
3. Quaranta, V. and G.E. Plopper. Integrins and laminins in tissue remodeling. *Kidney Int.*, 1997 (in press).
4. Tamura, R.N., Oda, D., Quaranta, V., Plopper, G., Lambert, R., Glaser, S., and J.C.R. Jones. Coating of titanium alloy with soluble laminin-5R promotes cell attachment and hemidesmosome assembly in gingival epithelial cells: Potential application to dental implants. *J. Periodontal Res.* 32:287-294, 1997.
5. Plopper, G.E., Domanico, S., Kioses, B., and V. Quaranta. Laminin-5 induced adhesion and migration in mammary epithelial cells: differential role of $\alpha 3\beta 1$ integrin in normal and transformed cell types. Manuscript submitted, 1997.
6. Plopper, G.E., and V. Quaranta. Mammary epithelial cell migration on laminin-5 mediated by G proteins and integrin activation. Manuscript submitted, 1997.

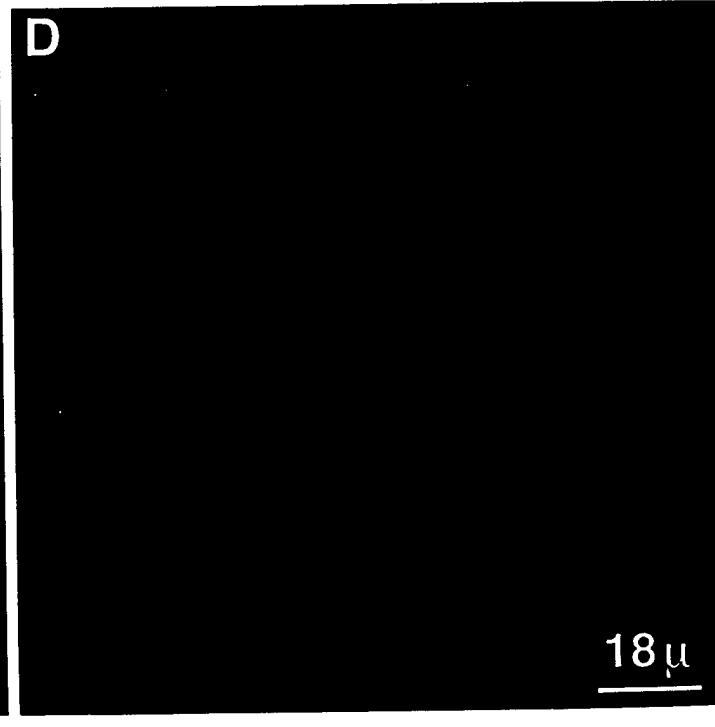
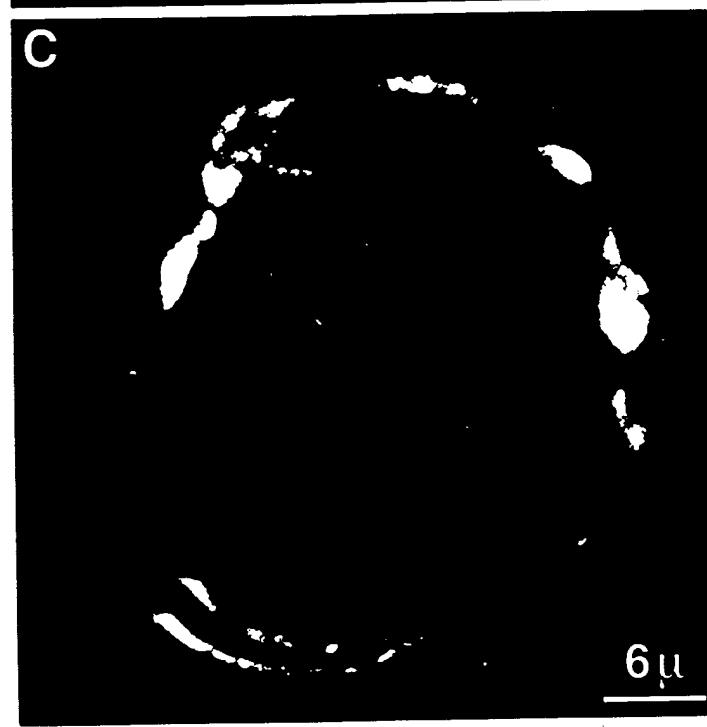
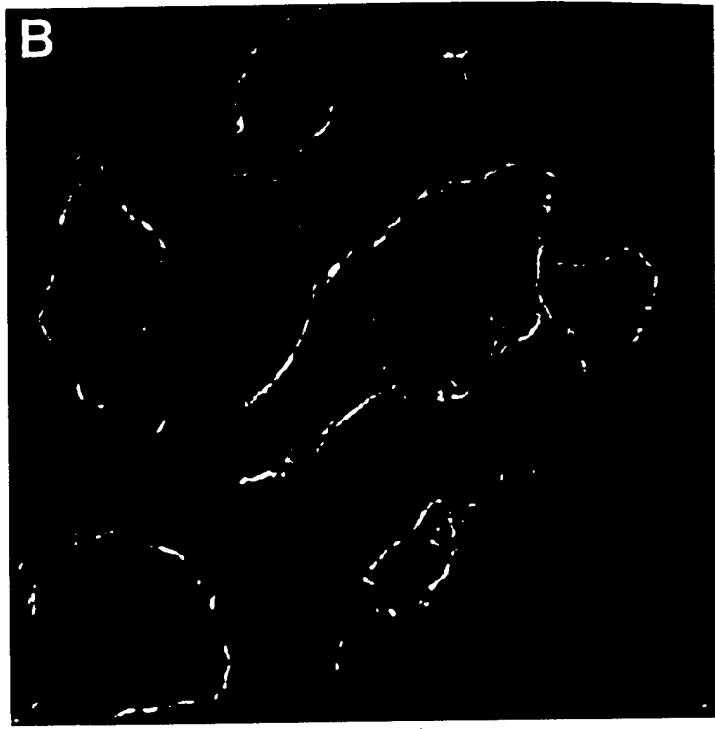
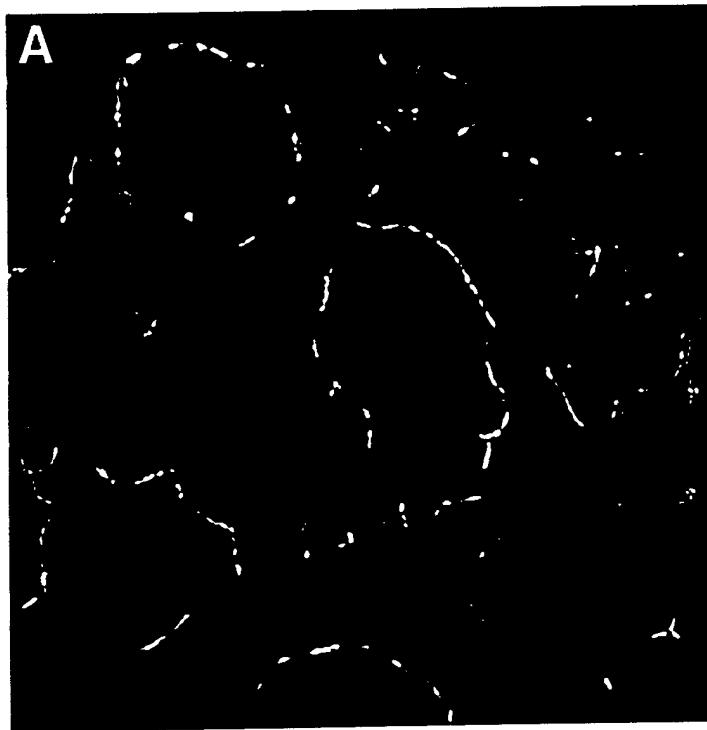
Abstracts:

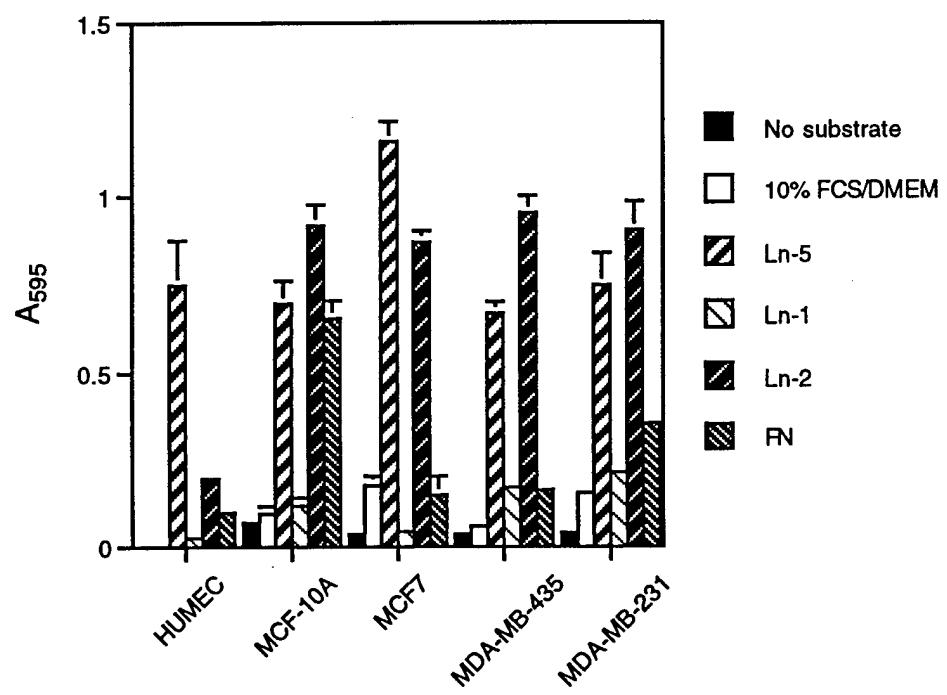
1. Hopkinson, S.B., S.E. Baker, M. Langhoffer, A. P. DePasquale, E.L. Stock, M. Hormia, J. Marzillier, G. Plopper, and V. Quaranta. Morphogenetic effects and utility in organ culture of soluble laminins: A novel biomaterial. *Mol. Biol. Cell* 5:180a, 1994.
2. Plopper, G., J. Falk-Marzillier, S. Glaser, M. Fitchmun, T. Romano, J.C.R. Jones, and V. Quaranta. Changes in conformation of laminin-5r induced by cell contact and defined by monoclonal antibodies. *Mol. Biol. Cell* 6:383a, 1995.
3. Falk-Marzillier, J., G. Plopper, M. Fitchmun, J.C.R. Jones, and V. Quaranta. The rapid adhesion promoting activity of laminin-5r is harbored in the $\alpha 3$ -chain. *Mol. Biol. Cell* 6:45a, 1995.
4. Baker, S.E., G. Plopper, V. Quaranta and J.C.R. Jones. Laminin-5 and Hemidesmosomes: Role of the α chain in nucleation of hemidesmosome assembly. *Mol. Biol. Cell* 6:192a, 1995.
5. Fitchmun, M.I., E. Marshall, G. Cruz, J. Falk-Marzillier, G. Plopper, J.C.R. Jones, and V. Quaranta. Mode of adsorption and orientation onto surfaces affect adhesion activity of laminin-5. *Mol. Biol. Cell* 7:58a, 1996 .

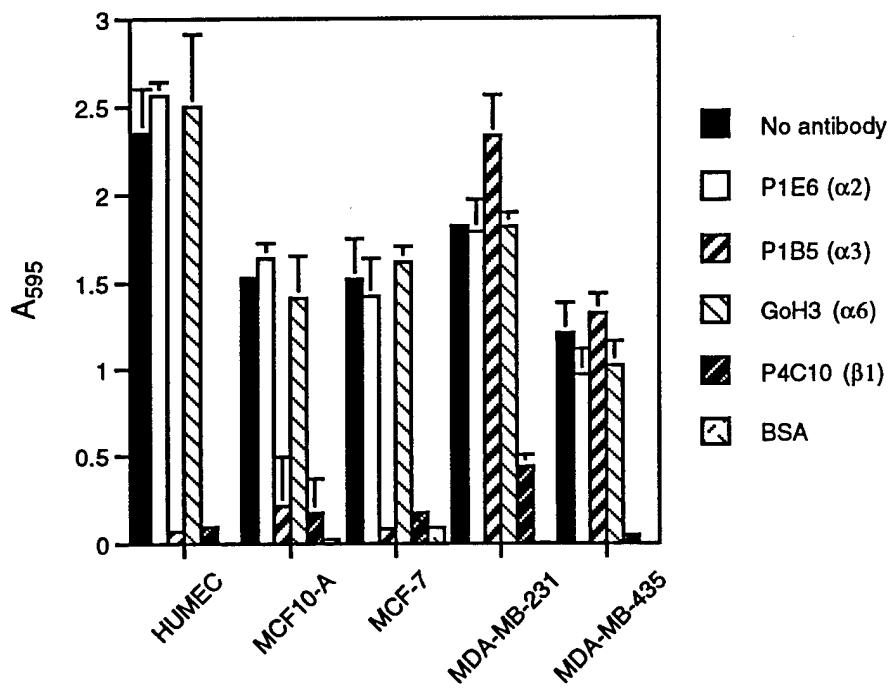
6. Baker, S.E., G. Plopper, M. Fitchmun, V. Quaranta, and J.C.R. Jones. Identification of a functional domain in laminin-5. Mol. Biol. Cell 7:86a, 1996.
7. Plopper, G. , W. Hwang, T. Sorensen, M. Fitchmun, V. Cirulli, and V. Quaranta. A G-protein signaling pathway controls migration of breast epithelial cells on laminin-5. Selected as "Hot Paper" at annual meeting of the American Society for Cell Biology, 1996.
8. Plopper, G. and V. Quaranta. Migration of invasive breast cancer cells is controlled by a G-protein linked receptor signaling pathway and integrin activation. Proc. Amer. Assoc. Cancer Res. 38:288, 1997.

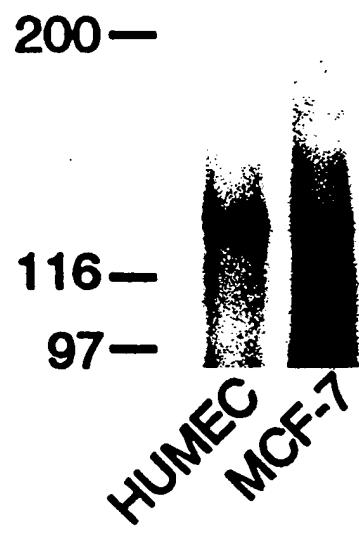
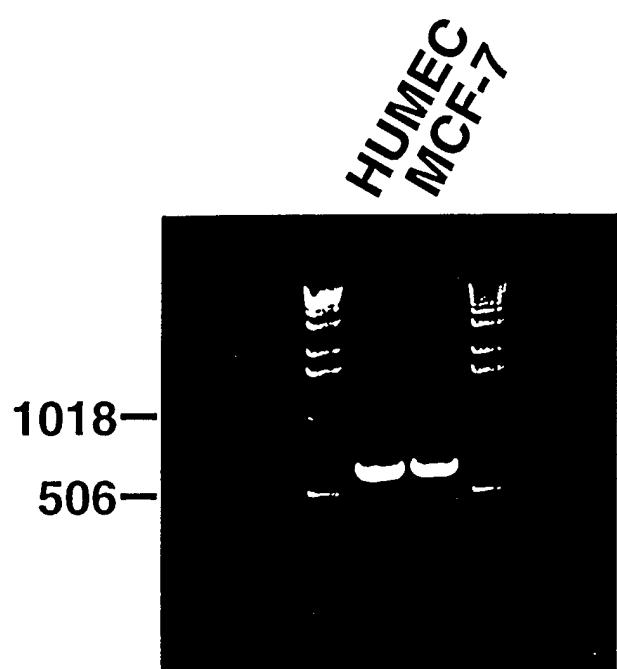
Personnel Receiving Pay:

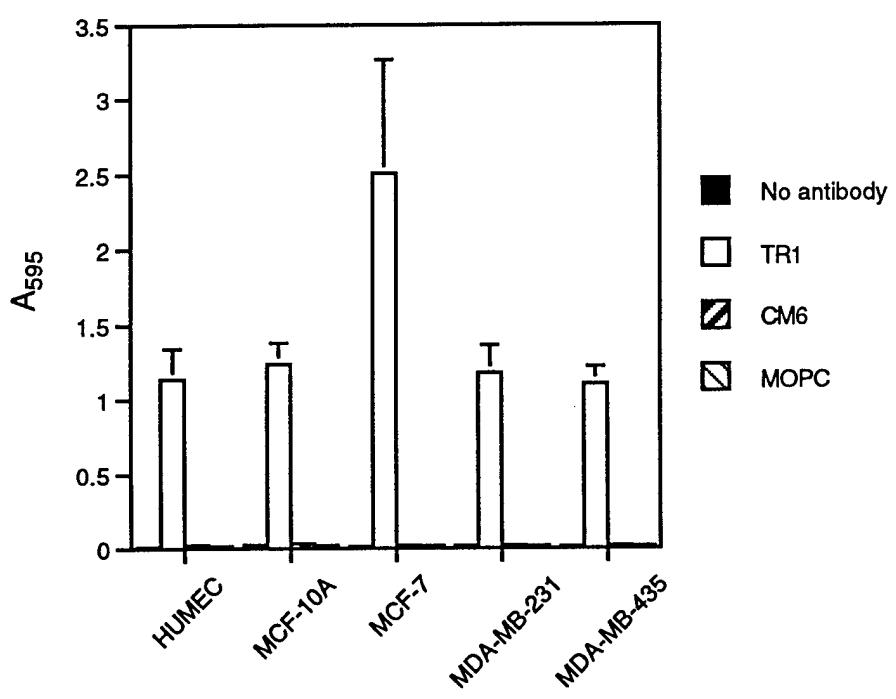
1. George Plopper, Ph.D., Research Associate, The Scripps Research Institute.

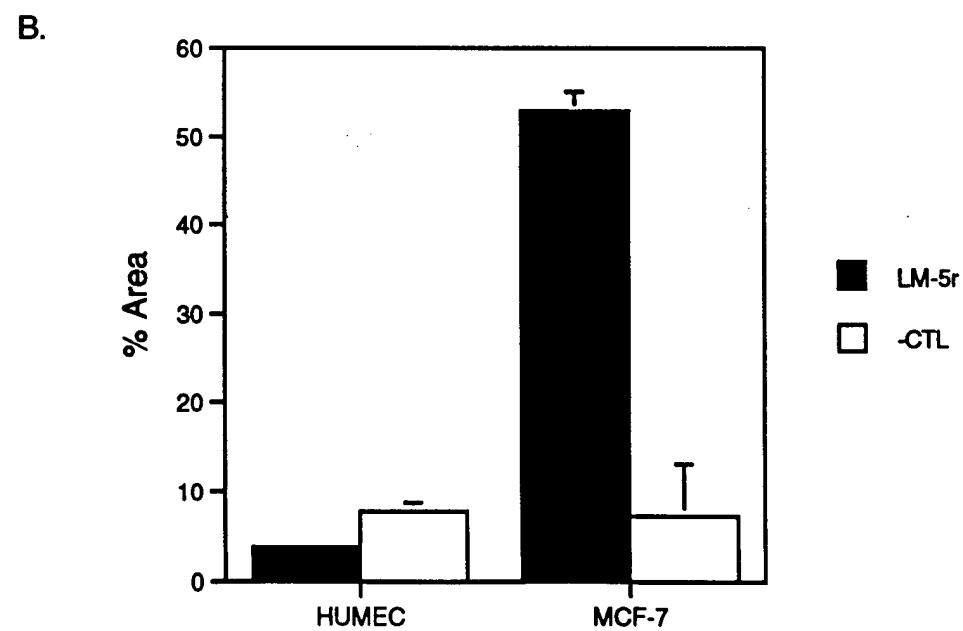
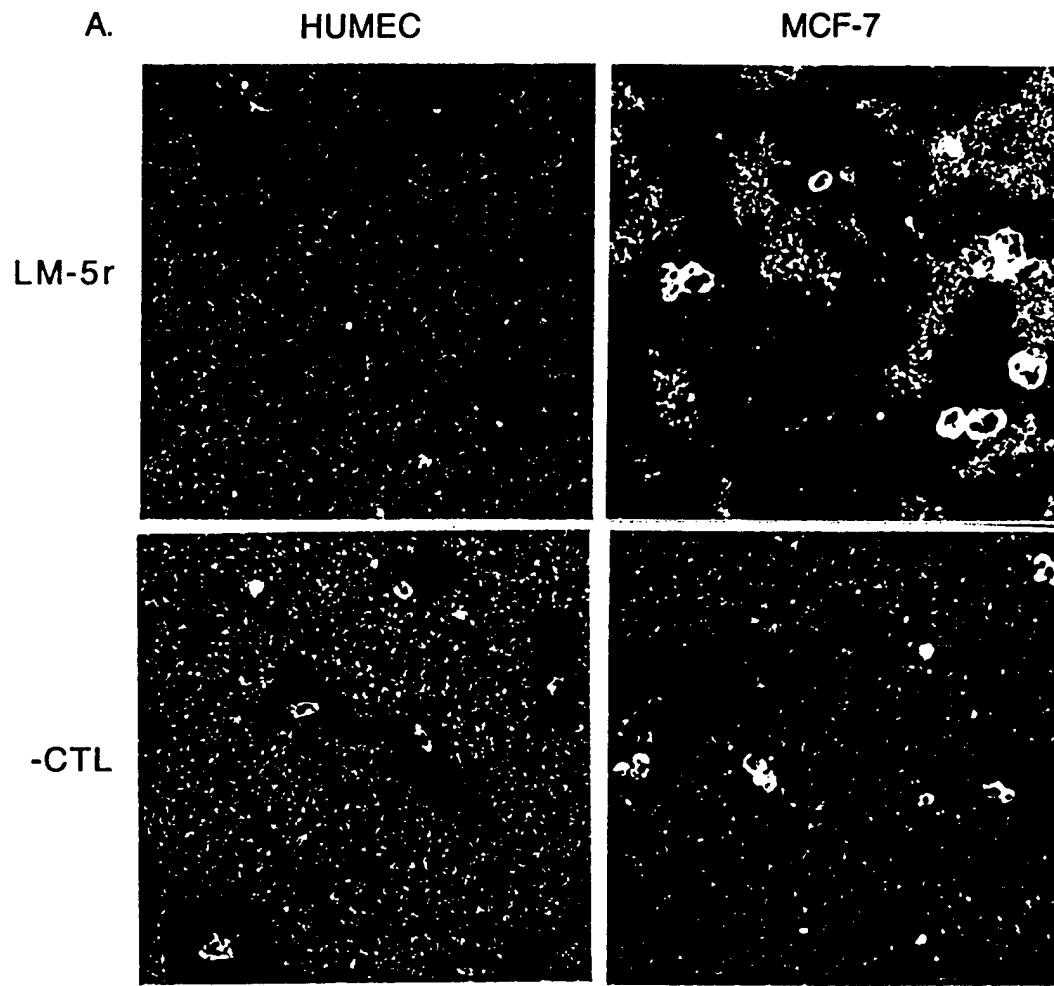


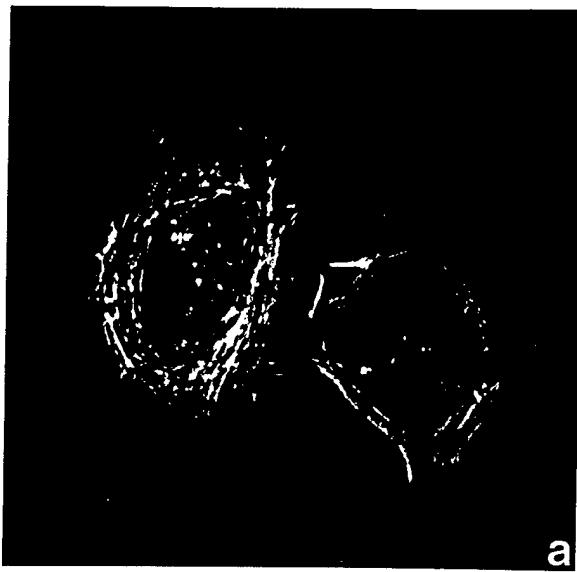




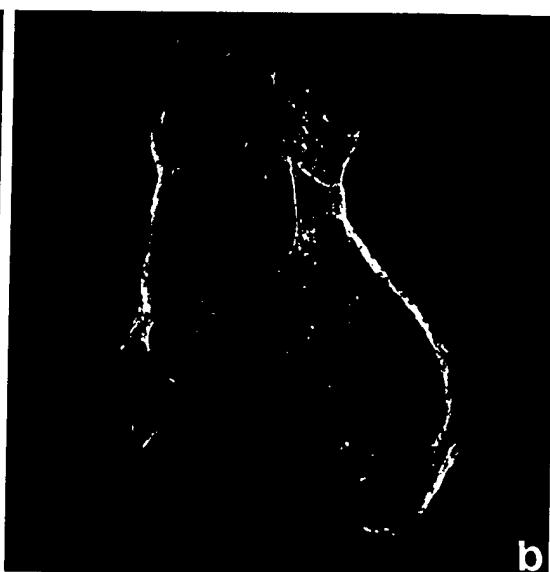




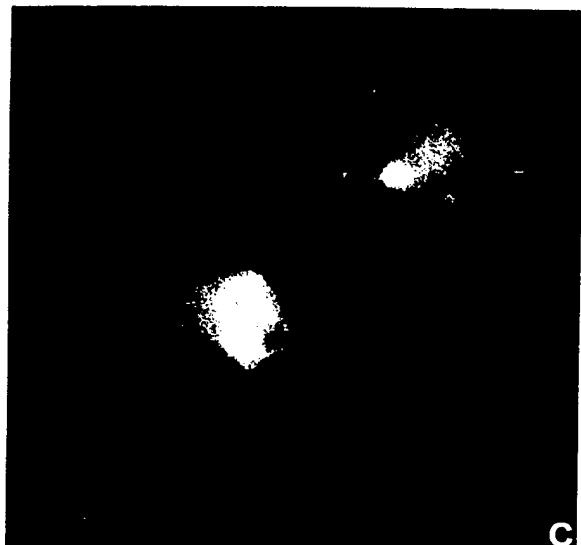




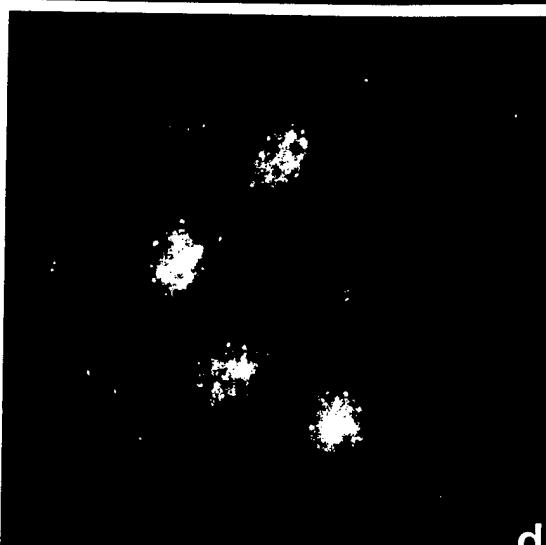
a



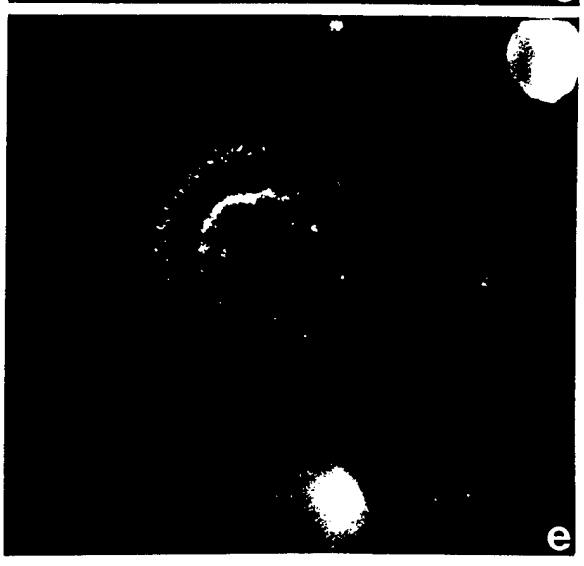
b



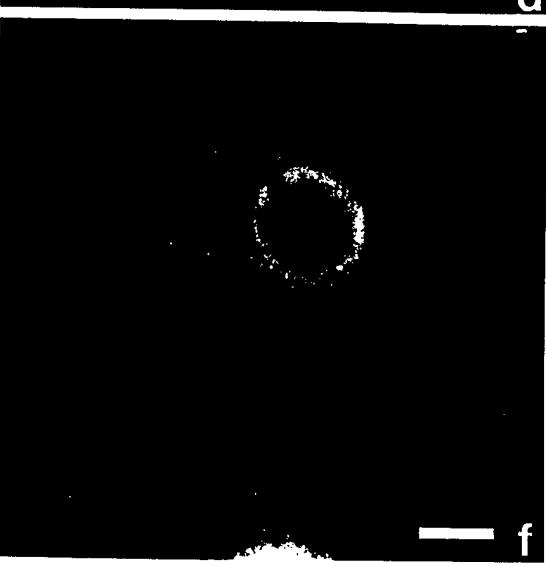
c



d



e



f

